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PRINCIPAL INVESTIGATOR: Anna M. Curatola, Ph.D.

CONTRACTING ORGANIZATION: New York University Medical Center
New York, New York 10010-2598

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13. ABSTRACT <i>(Maximum 200 words)</i> The $\alpha 6\beta 4$ integrin is a laminin 5 receptor expressed on the basal, basement membrane-apposed surface of ductal breast epithelial cells. In contrast to all other known integrins, $\alpha 6\beta 4$ is concentrated in hemidesmosomes, adhesive junctions which connect the basement membrane to the intracellular keratin cytoskeleton. In virtually all cases of human breast cancer analyzed, $\alpha 6\beta 4$ has been found to be diffusely distributed at the cell surface instead of being concentrated in hemidesmosomes. Our previous studies have indicated that $\alpha 6\beta 4$ promotes the assembly of hemidesmosomes by interacting, via a specific region of the large unique cytoplasmic domain of the $\beta 4$ subunit, with cytoskeletal elements of hemidesmosomes. We have observed that ligation of the EGF-R or EGF-R/Neu heterodimer promotes the association of the Src-family kinase Fyn with $\alpha 6\beta 4$ and begun to map the sequences of Fyn and $\beta 4$ required for the interaction. Ligation of the EGF-R or EGF-R/Neu heterodimer causes tyrosine phosphorylation of the $\beta 4$ tail and disassembly of hemidesmosomes. This phenomenon appears to be mediated by Fyn because a dominant negative version of the kinase inhibit tyrosine phosphorylation of the $\beta 4$ tail and disassembly of hemidesmosomes. These observations illustrate a mechanism by which overexpression of the EGF-R and/or Neu may lead to disassembly of hemidesmosomes in breast cancer. Future studies will address the relevance of this mechanism to tumor invasion.			
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INTRODUCTION

Cell-matrix interactions are likely to play an important role in breast tumorigenesis. Most human breast cancers arise from the transformation of ductal epithelial cells (Wellings et al., 1975; Dairkee et al., 1985; Rudland, 1987). Normal ductal epithelial cells rest on a basement membrane, to which they adhere tightly (Dairkee et al., 1985). The adhesion of normal breast epithelial cells to the basement membrane is thought to be important for the organization of the cytoskeleton and the consequent establishment of polarity. In addition, recent results indicate that normal breast epithelial cells receive signals from the basement membrane and these signals help them maintain a differentiated phenotype (Streuli et al., 1991). When compared to normal cells, breast carcinoma cells show a defective interaction with the basement membrane. First, like most carcinoma cells, they fail to assemble basement membrane components in an organized extracellular matrix, both *in vivo* and *in vitro* (Dulbecco et al., 1988; Petersen et al., 1992), and show cytoskeletal defects (Trask et al., 1990). Second, in contrast to normal breast epithelial cells, carcinoma cells do not arrest their growth when placed in a reconstituted basement membrane gel (Petersen et al., 1992). It is important to understand the molecular basis of these phenomena because they are likely to contribute to the ability of breast carcinoma cells to detach from the original tumor and invade adjacent tissues.

The molecular characterization of integrins provides a unique opportunity to examine the role of cell-matrix interactions in breast cancer. The integrins are a large family of adhesion receptors which bind to extracellular matrix components and, in some cases, to counter-receptors on other cells (Hynes, 1992). They consist of two distinct membrane-spanning subunits, α and β . At present we know of at least 9 homologous β subunits and 15 α subunits which can combine to form 21 receptors with distinct ligand binding specificities. Both the α and the β subunit (each ca. 140-200 kD m.w.) have a large extracellular portion, a transmembrane segment, and a short cytoplasmic domain. A notable exception is the $\beta 4$ subunit that has a large cytoplasmic domain. While the extracellular N-termini of α and β subunits associate to form the ligand binding pocket, the cytoplasmic domains of integrins interact with intracellular molecules.

The binding of integrins to extracellular matrix components promotes cell adhesion or migration, but ligation of integrins also results in intracellular signals which influence proliferation and differentiation (Giancotti, 1997). While contact with extracellular matrix components is required for the progression of normal cells through the cell cycle, a phenomenon called anchorage dependence, strong adhesion to an organized extracellular matrix seems to be able to limit cell proliferation (Giancotti and Ruoslahti, 1990) and promote differentiation (Streuli et al., 1991). The ability of integrins to modulate gene expression may help to explain the effects that the extracellular matrix has on proliferation and differentiation. The mechanisms by which integrins affect gene regulation are not completely understood, but likely depend on the ability of the cytoplasmic domains of

integrins to interact both with the cytoskeleton (Burridge et al., 1988) and with signaling molecules, such as the adaptor protein Shc (Mainiero et al., 1995; 1997; Wary et al., 1996) and Focal Adhesion Kinase (FAK) (Burridge et al., 1992).

Neoplastic cells are characterized by a number of adhesion abnormalities which may explain their ability to grow independently of the positive and negative control signals originating from the extracellular matrix (Giancotti and Mainiero, 1994). Virally transformed fibroblasts have a more rounded morphology in culture than their non-transformed counterparts. In addition, they often lack a cell surface fibronectin-containing pericellular matrix (Ruosahti, 1984). The defective fibronectin matrix of transformed fibroblasts may only partially be attributed to either decreased biosynthesis or increased proteolytic degradation of fibronectin, since the fibronectin secreted by transformed cells is regularly incorporated in the extracellular matrix by normal cells (Hayman et al., 1981). This suggests that transformed cells can not retain at their surface the fibronectin they produce, perhaps because of a defect in the integrin receptors. Several observations indicate that the expression and function of integrins are altered in neoplastic fibroblasts. While in normal fibroblasts the $\beta 1$ integrins, which include the $\alpha 5\beta 1$ fibronectin receptor, are clustered in focal adhesions (Chen et al., 1985; Damsky et al., 1985; Giancotti et al., 1986), transformed fibroblasts lack such structures and their $\beta 1$ integrins are found diffusely distributed over the cell surface (Giancotti et al., 1986; Chen et al., 1986). In addition, in fibroblasts transformed by tyrosine kinase oncogenes the $\beta 1$ subunit is found to be partially phosphorylated on a tyrosine residue (Hirst et al., 1986), a phenomenon which may reduce its ability to interact with the cytoskeleton (Tapley et al., 1989). Finally, the expression of $\alpha 5\beta 1$ and of another $\beta 1$ integrin, probably $\alpha 1\beta 1$, is suppressed in fibroblasts transformed by oncogenic viruses (Plantefaber and Hynes, 1989).

We have tested the hypothesis that changes in the level of expression or function of the $\alpha 5\beta 1$ fibronectin receptor contribute to the adhesive abnormalities of transformed fibroblasts by overexpressing this integrin in Chinese hamster ovary (CHO) cells (Giancotti and Ruosahti, 1990). The CHO cells have a transformed morphology, deposit little fibronectin in their pericellular matrix and are tumorigenic in vivo. As a result of the $\alpha 5\beta 1$ overexpression, the CHO cells accumulated a fibronectin matrix and became less migratory. These results indicate an inverse correlation between matrix assembly of fibronectin and cell migration and suggest that the loss of fibronectin matrix and the increased invasive ability of transformed fibroblasts can be both brought about by a reduced expression or function of $\alpha 5\beta 1$. Interestingly, the CHO cells overexpressing $\alpha 5\beta 1$ were also found to be more anchorage dependent than the controls and were not able to form subcutaneous tumors in nude mice. K562 leukemia cells selected for high level expression of $\alpha 5\beta 1$ show a similar normalization of growth properties (Symington, 1990). Conversely, CHO cells selected for their low levels of $\alpha 5\beta 1$ expression are more tumorigenic than unselected cells (Schreiner et al., 1991). Thus, it appears that changes in the level of expression or activity of certain integrins may not only be responsible for the adhesive defects of neoplastic cells but may also contribute to

their unregulated growth. Taken together, these observations suggest that the role of integrins in tumorigenesis is twofold: first, integrins mediate stable adhesion or migration onto extracellular matrix components and changes in their level of expression and function may, therefore, contribute to tumor invasion. Second, integrins transmit signals from the extracellular matrix to the cell interior and these signals affect cellular growth and differentiation. Therefore changes in integrins may contribute to the unrestrained growth and lack of differentiation of neoplastic cells.

Although the adhesive phenotype of breast carcinoma cells is less well known than that of neoplastic fibroblasts, certain rules learned from the analysis of virally transformed fibroblasts seem to also apply to these cells. For example, breast carcinoma cells fail to assemble basement membrane components in an organized extracellular matrix (Dulbecco et al., 1988; Petersen et al., 1992) and show enhanced ability to grow when confronted with a reconstituted basement membrane gel (Petersen et al., 1992). Immunohistochemical studies have indicated that the expression levels of the $\alpha 2\beta 1$ collagen/laminin receptor, the $\alpha 5\beta 1$ fibronectin receptor and the $\alpha 6\beta 4$ integrin are altered in human carcinomas of the breast (Zutter et al., 1990; Koukoulis et al., 1991; Natali et al., 1992). In addition, while integrins are generally polarized at the basal or baso-lateral surface in normal breast epithelium, the integrins expressed in breast carcinoma cells are diffusely distributed over the cell surface (Zutter et al., 1990; Koukoulis et al., 1991; Natali et al., 1992). It is our hypothesis that these phenomena contribute to the ability of breast carcinoma cells to detach from the original tumor and invade the adjacent tissues.

BODY

We have initially focused on establishing a transgenic mouse model system in which to investigate the role of integrin defects in breast cancer progression. To this end, we have examined transgenic mice carrying either an activated or a normal form of the N-Ras oncogene under the control of the Mammary Tumor Virus Long Terminal Repeat (MMTV-LTR) promoter. These mice, similar to mice carrying activated forms of the H-Ras or Neu oncogenes, develop mammary carcinomas with a high frequency during the first few months of their life (Sinn et al., 1987; Muller et al., 1988; Mangues et al., 1992; R. Mangues & A. Pellicer, Department of Pathology, N.Y.U. School of Medicine, unpublished results). The tumors which develop often consist of areas of different levels of histological differentiation and thus can provide an insight to the process of primary breast tumor progression.

Immunohistochemical analyses conducted on these tumors revealed a significant loss of laminin staining. The $\alpha 6$ and $\beta 4$ subunits were not downregulated, but lacked polarization; some basal staining could be seen in better differentiated tumor areas. The $\beta 1$ staining was similarly no longer polarized. The $\alpha 2$ and $\alpha 3$ subunits were also diffusely distributed at the tumor cell surface with an

apparent increased intensity of $\alpha 2$ staining. The $\alpha 5$ and αv subunits were not expressed in the tumors.

To determine if the changes in integrin expression *in vivo* were a direct result of Ras or due to other genetic changes which occur during tumor progression, the effect of the expression of the N-Ras oncogene on integrin expression in a normal murine breast cell line was investigated. Cell surface labeling and immunoprecipitation analysis indicated that the breast epithelial cell lines acutely transformed by N-Ras expressed an integrin repertoire indistinguishable from that of control untransformed cell lines. Moreover the level of expression of individual integrin subunits in N-Ras expressing cell lines was unchanged as compared to the controls. Similar results were obtained by comparing breast carcinoma cells overexpressing the Erb2/Neu with cell lines not expressing Erb2/Neu. Taken together, these findings indicate that Ras and ERb2/Neu cannot directly affect integrin expression and suggest that the changes in integrin expression observed in the transgenic model were not a direct consequence of oncogene action, but were caused by additional genetic changes associated with tumor progression.

Considering the altered cell surface distribution of $\alpha 6\beta 4$ observed by us in the mammary tumors of transgenic mice and by others in human breast carcinomas, we decided to focus on examining the mechanisms by which neoplastic transformation could alter the subcellular distribution of this integrin.

Association of $\alpha 6\beta 4$ with cytoskeletal and signaling molecules

In contrast to all the other known α and β subunit cytoplasmic domains, which are relatively short, the intracellular portion of $\beta 4$ subunit measures over 1000 amino acids in length and contains, in its C-terminal half, two pairs of type III fibronectin-like repeats separated by a 142 amino acid Connecting Segment (Hogervorst et al., 1990; Suzuki and Naitoh, 1990). While $\beta 1$ and αv -subunit containing integrins interact with the actin cytoskeleton and localize to focal adhesions, $\alpha 6\beta 4$ is found concentrated at hemidesmosomes both in cultured cells and *in vivo* (Carter et al., 1990; Stepp et al., 1990), suggesting that the $\beta 4$ tail specifies association with the hemidesmosomal cytoskeleton.

Hemidesmosomes are complex adhesive junctions which link the basement membrane to the intracellular keratin cytoskeleton and are exclusively found in the basal cell layer of stratified and transitional epithelia (Borradori and Sonnenberg, 1996). Gene transfer studies in cultured cells have provided evidence that the unique cytoplasmic domain of $\beta 4$, and specifically a region which encompasses the first pair of type-III fibronectin-like modules and the Connecting Segment, is required for association of $\alpha 6\beta 4$ with the hemidesmosomal cytoskeleton (Spinardi et al., 1993). Subsequent studies have indicated that ligation of $\alpha 6\beta 4$ activates an integrin-associated kinase and causes phosphorylation of the $\beta 4$ cytoplasmic domain at multiple tyrosine residues (Mainiero et al., 1995). Phosphorylation of a Tyrosine Activation Motif (TAM) located in the Connecting Segment is likely to be

required for association with the hemidesmosomal cytoskeleton, because mutations at either one of its two constituent tyrosine residues abolish the incorporation of $\alpha 6\beta 4$ in hemidesmosomes (Mainiero et al., 1995). The ability of a truncated $\beta 4$ subunit to exert a dominant negative effect on hemidesmosome assembly without inhibiting initial adhesion to laminin 5 (Spinardi et al., 1995) and the absence of hemidesmosomes in the skin of $\alpha 6$ and $\beta 4$ knock-out mice (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996) suggest that $\alpha 6\beta 4$ plays a crucial role in the assembly of hemidesmosomes and their linkage to the keratin filament system.

Effect of Epidermal Growth Factor on the intercellular functions of $\alpha 6\beta 4$ integrin.

Prompted by the prominent role of EGF and TGF- α in controlling epithelial cell growth and migration and by the coincident expression of $\alpha 6\beta 4$ and EGF-R in many epithelial tissues including the breast epithelium, we have examined the effect of EGF-R activation on the intracellular functions of $\alpha 6\beta 4$. Experiments of immunoblotting with anti-phosphotyrosine (anti-P-Tyr) antibodies and immunoprecipitation followed by phosphoamino acid analysis and phosphopeptide mapping showed that activation of the EGF-R causes phosphorylation of the $\beta 4$ subunit at multiple tyrosine residues. Interestingly, immunoprecipitation experiments indicated that stimulation with EGF does not result in association of $\alpha 6\beta 4$ with the adaptor protein Shc. In contrast, EGF can partially suppress the recruitment of Shc to ligated $\alpha 6\beta 4$. Furthermore, immunofluorescent analysis revealed that EGF treatment does not induce increased assembly of hemidesmosomes, but instead causes a deterioration of these adhesive structures without affecting initial adhesion to laminins. Finally, Boyden chamber assays indicated that exposure to EGF results in upregulation of $\alpha 6\beta 4$ -mediated cell migration toward laminins. Taken together, these results indicate that EGF-dependent signals have a complex effect on $\alpha 6\beta 4$ function: they cause tyrosine phosphorylation of $\beta 4$ without promoting the association of Shc, induce disassembly of hemidesmosomes, and upregulate cell migration on laminins (Mainiero et al., 1996). It is possible that the ability of activated EGF-R to coordinately disassemble hemidesmosomes and upregulate $\alpha 6\beta 4$ -dependent cell migration play a role during tumor progression.

Since the consequences of EGF-mediated and ligand-induced tyrosine phosphorylation of $\beta 4$ are distinct, it is likely that the activation of EGF-R and ligation of $\alpha 6\beta 4$ cause phosphorylation of distinct sites in the $\beta 4$ tail. Although we cannot formally exclude that the EGF-mediated and ligand-induced phosphorylation of $\beta 4$ are mediated by a single tyrosine kinase differentially regulated by the two stimuli, it is more likely that they are mediated by two distinct tyrosine kinases. Since the immunopurified EGF-R does not efficiently phosphorylate $\beta 4$ *in vitro*, it is our hypothesis that the EGF-R does not directly phosphorylate $\beta 4$ *in vivo* but rather activates a signaling pathway that results in its phosphorylation.

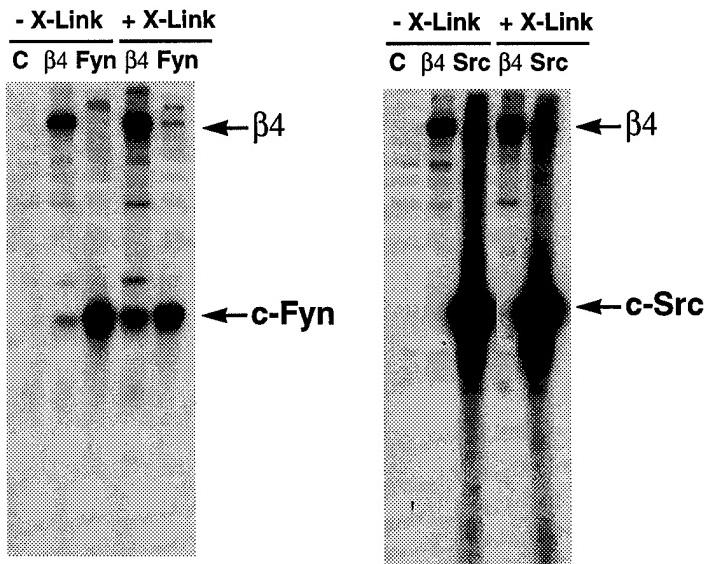


Fig. 1. Association of $\alpha 6\beta 4$ with c-Fyn. 293-T cells were transiently transfected with constructs encoding $\alpha 6$ and $\beta 4$ in combination with either c-Fyn (left) or c-Src (right). After stimulation with control (- X-Link) or anti- $\beta 4$ (+ X-Link) Mabs, the cells were extracted and immunoprecipitated with the indicated antibodies. The immune complexes were subjected to in vitro kinase assay and the gels treated with alkali to remove radioactive phosphate bound to serine and threonine residues.

To identify tyrosine kinases capable of phosphorylating $\alpha 6\beta 4$, we have performed immune-complex kinase assays in 293-T cells transiently transfected with constructs encoding $\alpha 6$ and $\beta 4$ in combination with each one of various tyrosine kinases. The results indicated that the Src-family kinase Fyn can associate with $\alpha 6\beta 4$ and phosphorylate the $\beta 4$ subunit. In contrast, Src, Lck, and Jak-1 did not associate with $\alpha 6\beta 4$, but were able to induce phosphorylation of $\beta 4$ (Fig. 1 and data not shown). The ability of several overexpressed tyrosine kinases to phosphorylate $\beta 4$ is not surprising, because the $\beta 4$ tail contains multiple potential tyrosine phosphorylation sites. Antibody-mediated cross-linking of $\alpha 6\beta 4$ increased the kinase activity of the integrin-associated fraction of Fyn (Fig. 1), without inducing further recruitment of the kinase (data not shown). These observations indicate that upon overexpression Fyn can combine with $\alpha 6\beta 4$ and phosphorylate the $\beta 4$ tail.

The association of $\alpha 6\beta 4$ with Fyn is likely to be mediated by the $\beta 4$ tail because $\alpha 6\beta 1$ does not interact with Fyn (data not shown). The $\beta 4$ tail contains three regions potentially capable of interacting with Fyn: a membrane-proximal Cys-X-X-Cys motif which could interact with a similar motif in the unique N-terminal segment of Fyn (a similar mechanism underlies the association of the T cell coreceptors CD4 and CD8 with Lck), a Pro-X-X-Pro sequence in the N-terminal portion of Connecting segment which could bind to the SH3 domain of Fyn, and the TAM in the C-terminal portion of Connecting Segment which may interact with the unique N-terminal segment of Fyn (this portion of Fyn has been shown to interact, in a phosphorylation independent manner, with immune receptor TAMs). To gain insight to the mechanism of Fyn association with $\alpha 6\beta 4$, we have used a mutational approach. The wild-type $\beta 4$ subunit and several of the deletion mutants illustrated in Fig. 2 A were introduced together with $\alpha 6$ and Fyn in 293-T cells. The results of coimmunoprecipitation analysis indicated that a total and an almost total deletion of the $\beta 4$ tail suppress the association of Fyn (constructs B and L) (Fig. 2 B).

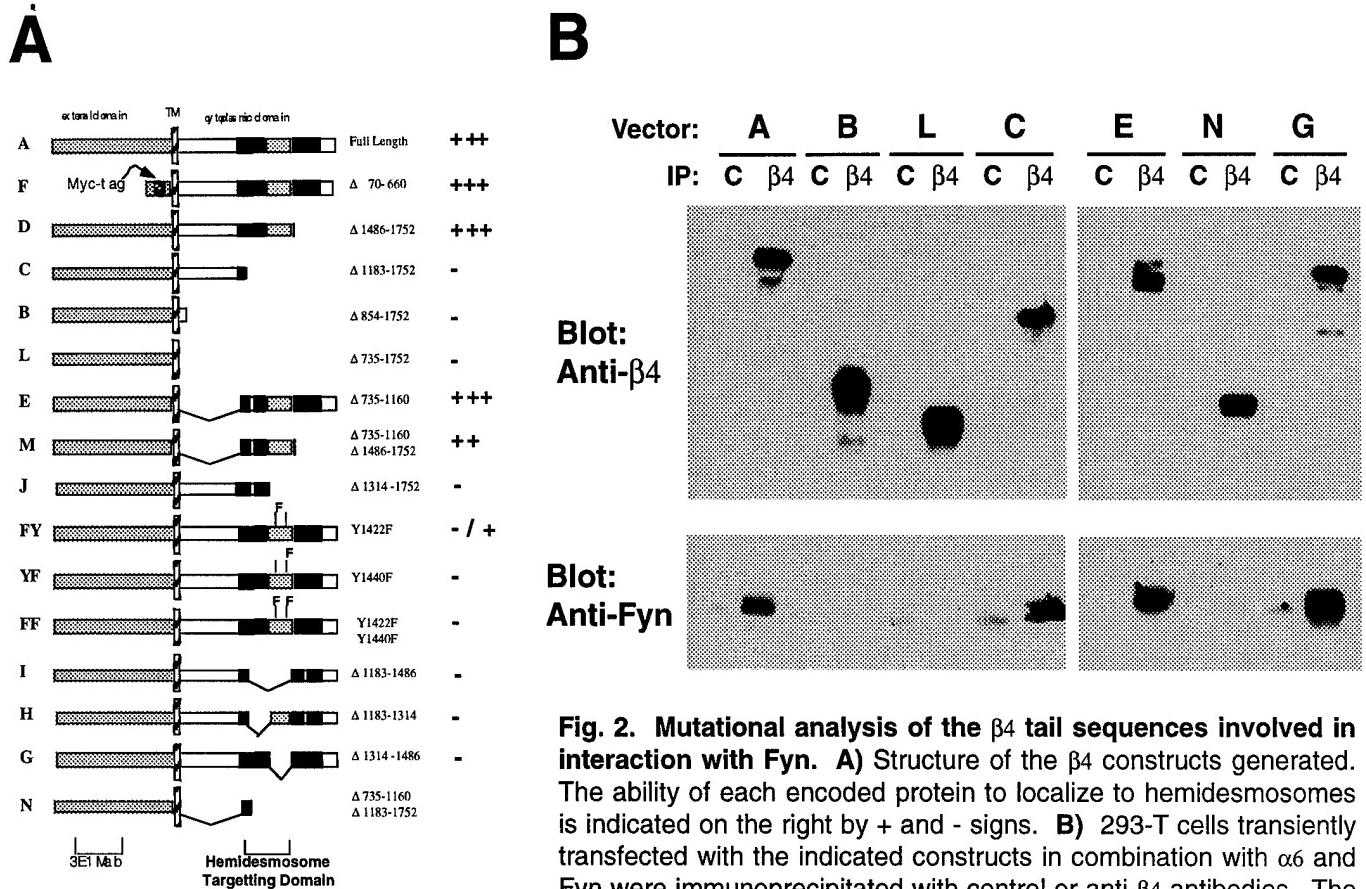


Fig. 2. Mutational analysis of the β 4 tail sequences involved in interaction with Fyn. **A)** Structure of the β 4 constructs generated. The ability of each encoded protein to localize to hemidesmosomes is indicated on the right by + and - signs. **B)** 293-T cells transiently transfected with the indicated constructs in combination with α 6 and Fyn were immunoprecipitated with control or anti- β 4 antibodies. The immunoprecipitates were probed by immunoblotting with anti- β 4 exo (top) and anti-Fyn antibodies (bottom).

In contrast, neither a deletion of the N-terminal region (aa 735-1160) nor one of the C-terminal region of the β 4 tail (aa 1183-1752) abolished association of Fyn (C and E). Since the two latter constructs have a short region of overlap, we constructed a β 4 mutant containing the region of overlap as its only cytoplasmic segment (N). This mutant was not able to combine with Fyn (Fig. 2 A). It is likely that this small segment, which is part of the first type III Fn-like module, is required, but not sufficient for association with Fyn, or needs to be spaced from the plasma membrane. Further mutagenesis is being conducted to precisely map the β 4 sequences involved in interaction with Fyn. The more unlikely possibility that the β 4 tail contains two distinct sequences interacting with Fyn is also being considered.

We next wanted to identify the region of Fyn mediating the interaction with the β 4 tail. Src family kinases contain at least three domains that can mediate protein-protein interactions: the unique N-terminal segment, the SH3 domain, and the SH2 domain. While the unique N-terminal segment and the SH2 domain mediate interaction with upstream signal transducers, such as receptor tyrosine

kinases and immune receptors, the SH3 domain is involved in binding to substrates. The interaction of Fyn with $\alpha 6\beta 4$ does not appear to be phosphorylation dependent and is therefore unlikely to be mediated by the SH2 domain. In any event, we constructed three mutant forms of Fyn: one lacking of the SH3 domain (Δ SH3), one carrying an inactivating point mutation in the SH2 domain (SH2 $^{-}$), and a chimera consisting of the unique N-terminus (13 aa) of Fyn fused to the SH3, SH2 and kinase domain of Src (Fyn13N-Src). As shown in Fig. 3, all mutant forms of Fyn, including Fyn13N-Src, but not Src combined with $\alpha 6\beta 4$ upon transfection in 293-T cells. These results indicate that the unique N-terminus of Fyn is required and sufficient for interaction with $\alpha 6\beta 4$.

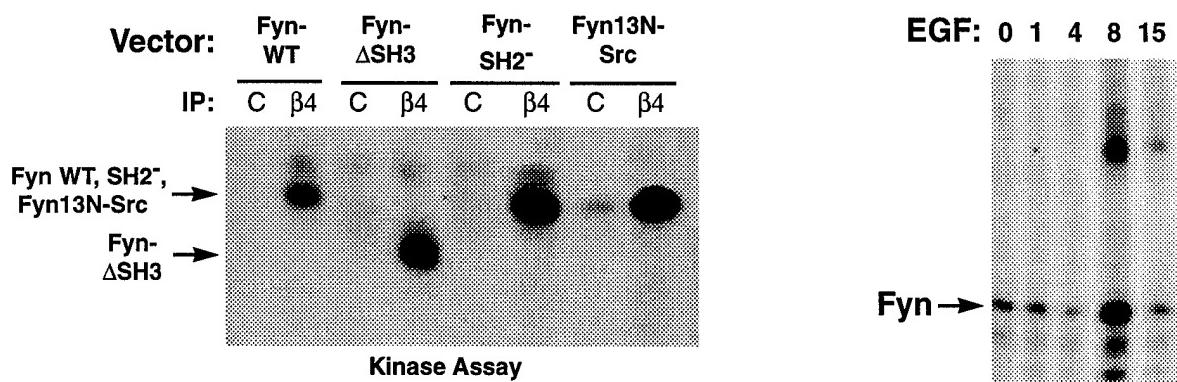


Fig. 3. The association of Fyn with $\beta 4$ is mediated by the unique N-terminus of Fyn. 293-T cells were transiently transfected with $\alpha 6$ and $\beta 4$ in combination with each one of the indicated Fyn constructs. The cells were immunoprecipitated with control or anti- $\beta 4$ antibodies and the precipitates were subjected to immune-complex kinase assay.

Fig. 4. EGF-induced activation of Fyn. Cells were stimulated with 50 ng/ml EGF for the indicated times and immunoprecipitated with anti-Fyn antibodies. The precipitates were subjected to immune-complex kinase assay.

Since coimmunoprecipitation experiments indicated that $\alpha 6\beta 4$ is not constitutively associated with endogenous Fyn in A431 cells, HaCat keratinocytes, and 293 T cells stably transfected with $\beta 4$ cDNA, we wondered if Fyn played a role in EGF-induced phosphorylation of $\beta 4$ and associated with $\alpha 6\beta 4$ only in response to EGF treatment. In accordance with this hypothesis, preliminary experiments indicated that a concentration of EGF, which is able to induce phosphorylation of $\beta 4$, activates Fyn in a time-dependent manner (Fig. 4). Subsequent coimmunoprecipitation experiments indicated that, upon treatment with EGF, $\alpha 6\beta 4$ forms a complex with endogenous Fyn in 293-T cells stably expressing $\beta 4$ (Fig. 5 A), A431 cells, and HaCat keratinocytes (data not shown). In contrast, various $\beta 1$ integrins did not form a complex with the activated EGF-R and Fyn (data not shown). Immunoblotting with anti-phosphotyrosine antibodies revealed that a 180 kD mol. wt. protein formed a complex with $\alpha 6\beta 4$ upon EGF stimulation (Fig. 5 A).

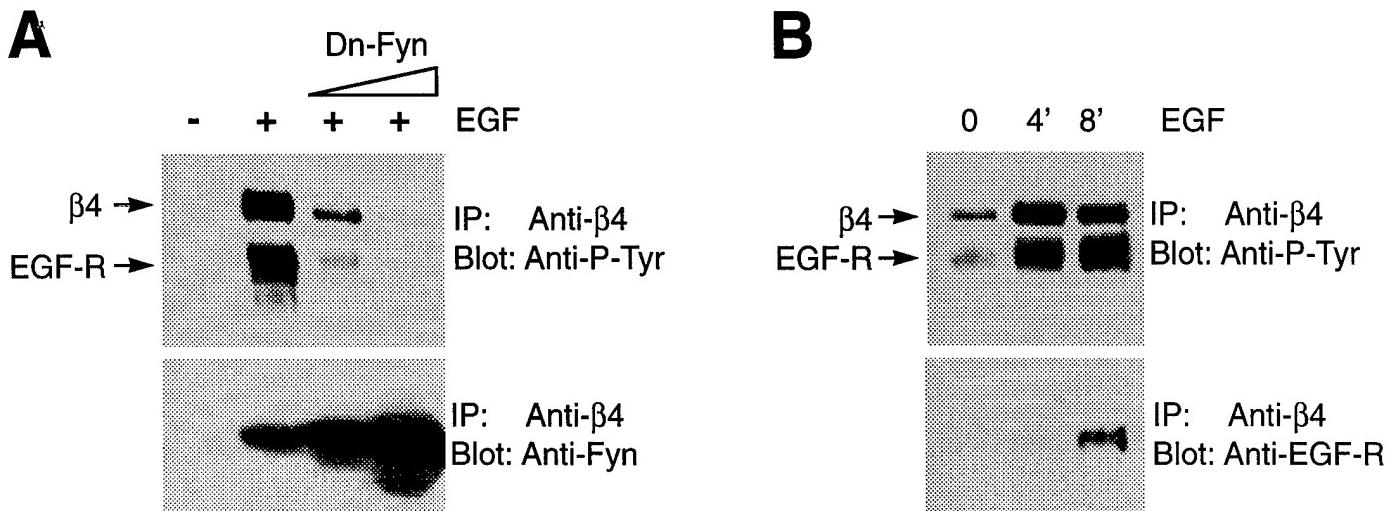


Fig. 5. Ligation of the EGF-R induces recruitment of Fyn and tyrosine phosphorylation of $\beta 4$. **A)** 293-T- $\beta 4$ cells were either left untransfected or transiently transfected with 3 and 6 μ g of dominant negative (Dn), kinase-dead Fyn, then either left untreated or stimulated with EGF, extracted, and immunoprecipitated with anti- $\beta 4$ antibodies. The top of the blot was probed with anti-phosphotyrosine (anti-P-Tyr) and the bottom with anti-Fyn antibodies. Note that endogenous Fyn (second lane) and increasing concentrations of Dn Fyn (third and fourth lanes) combine with $\alpha 6\beta 4$ in response to EGF. **B)** A431 cells were treated with EGF for the indicated minutes, extracted in Triton X-100, and immunoprecipitated with anti- $\beta 4$ Mab. Parallel blots were probed with anti-P-Tyr (top) and anti-EGF-R (bottom) antibodies. Note that at 4 minutes the fraction of EGF-R associated with $\alpha 6\beta 4$ is small, but highly phosphorylated.

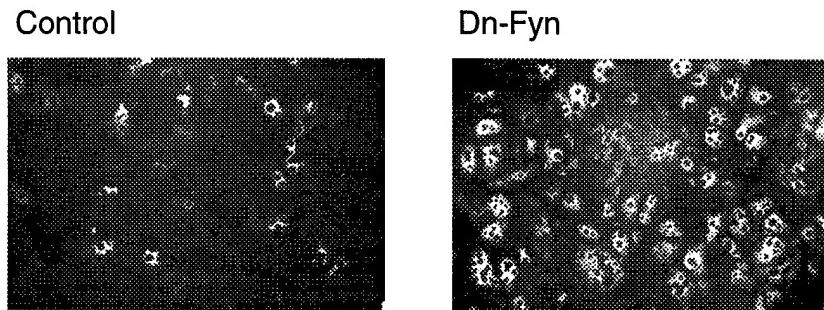


Fig. 6. Effect of dominant negative Fyn on hemidesmosomes. Immunofluorescent staining of hemidesmosomes in 804G cells stably transfected with control vector or vector encoding Dn Fyn.

Immunoblotting with anti-EGF-R antibodies indicated that this protein corresponded to the autophosphorylated EGF-R (Fig. 5 B). These results suggest that the activated EGF-R may recruit Fyn to the plasma membrane, thereby facilitating its interaction with $\alpha 6\beta 4$. In accordance with this hypothesis, we have observed that increasing amounts of dominant negative (kinase-dead) Fyn suppress the phosphorylation of $\beta 4$ induced by EGF (Fig. 5 A). Since the phosphorylation of $\beta 4$ induced by EGF has a negative effect on hemidesmosome assembly, we have tested the effect of dominant negative Fyn on hemidesmosome assembly. Interestingly, expression of dominant negative Fyn led to increased assembly of hemidesmosomes in rat 804G cells (Fig. 6). These results are consistent with the hypothesis that Fyn is the tyrosine kinase which downregulates the intracellular functions of $\alpha 6\beta 4$ in response to EGF stimulation.

The observation that the EGF-R and its close relative Erb2/Neu are frequently overexpressed in squamous carcinomas raises the possibility that either the EGF-R or Erb2/Neu or both affect the association of $\alpha 6\beta 4$ with the hemidesmosomal cytoskeleton in breast cancer cells and these events may contribute to tumor invasion. In order to examine this hypothesis, we will compare the ability of breast cell lines expressing different levels of EGF-R or Erb2/neu to invade through matrigel in a Boyden chamber assay. If we find a correlation between increased expression of the EGF-R or Erb2/Neu and invasive ability *in vitro*, we will perform tumorigenicity assays in nude mice. Finally, we will introduce dominant negative Fyn in at least three distinct cell lines overexpressing the EGF-R or Erb2/Neu to examine if it suppresses the invasive ability of these cells, both *in vitro* and *in vivo*. As a control, the cells will be transfected with vectors encoding kinase-dead versions of Fyn carrying a second mutation which inactivates its interaction with the growth factor receptor or $\alpha 6\beta 4$. These experiments should help to understand if the interaction of EGF-R family members with $\alpha 6\beta 4$ mediated by Fyn plays a role in tumor invasion.

CONCLUSIONS

The above described studies indicate that the expression, and possibly the function, of several integrins involved in adhesion to the basement membrane is altered during the *in vivo* progression of breast cancer in the N-Ras transgenic mouse model. The $\alpha 6\beta 4$ and, to a minor extent, the $\alpha 2\beta 1$ integrin are upregulated and diffusely distributed at the tumor cell surface in the primary lesions. These events are accompanied by a loss of laminin staining indicative of defective basement membrane deposition. The $\alpha 3\beta 1$ integrin is diffusely distributed, but not upregulated. Since transfection of N-ras or overexpression of Erb2/Neu in breast epithelial cells does not produce the changes in integrin expression detected *in vivo*, it is likely that these changes occur as a result of tumor progression independently of a direct action of N-ras. We have thus focused on the mechanism by which tumorigenesis may lead to changes in the cell surface distribution and function of $\alpha 6\beta 4$. Our results indicate that the activated EGF-R combines sequentially with Fyn and $\alpha 6\beta 4$. Fyn then phosphorylates the $\beta 4$ tail leading to disassembly of hemidesmosomes and possibly increased migratory activity. Future experiments will be addressed at determining if the EGF-R family member Erb2, which is often mutated and overexpressed in breast cancer, also combines with $\alpha 6\beta 4$ and if this event contributes to breast cancer progression.

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Signal transduction by the $\alpha_6\beta_4$ integrin: distinct β_4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes

Fabrizio Mainiero, Angela Pepe,
Kishore K.Wary, Laura Spinardi¹,
Moosa Mohammadi², Joseph Schlessinger²
and Filippo G.Giancotti³

Departments of Pathology and ²Pharmacology, Kaplan Comprehensive Cancer Center, New York University School of Medicine, New York, NY 10016, USA

¹Present address: DIBIT, Istituto Scientifico S. Raffaele, Milan, 20132, Italy

³Corresponding author

We have examined the mechanism of signal transduction by the hemidesmosomal integrin $\alpha_6\beta_4$, a laminin receptor involved in morphogenesis and tumor progression. Immunoprecipitation and immune complex kinase assays indicated that antibody- or laminin-induced ligation of $\alpha_6\beta_4$ causes tyrosine phosphorylation of the β_4 subunit in intact cells and that this event is mediated by a protein kinase(s) physically associated with the integrin. Co-immunoprecipitation and GST fusion protein binding experiments showed that the adaptor protein Shc forms a complex with the tyrosine-phosphorylated β_4 subunit. Shc is then phosphorylated on tyrosine residues and recruits the adaptor Grb2, thereby potentially linking $\alpha_6\beta_4$ to the ras pathway. The β_4 subunit was found to be phosphorylated at multiple tyrosine residues *in vivo*, including a tyrosine-based activation motif (TAM) resembling those found in T and B cell receptors. Phenylalanine substitutions at the β_4 TAM disrupted association of $\alpha_6\beta_4$ with hemidesmosomes, but did not interfere with tyrosine phosphorylation of Shc and recruitment of Grb2. These results indicate that signal transduction by the $\alpha_6\beta_4$ integrin is mediated by an associated tyrosine kinase and that phosphorylation of distinct sites in the β_4 tail mediates assembly of the hemidesmosomal cytoskeleton and recruitment of Shc/Grb2.

Keywords: hemidesmosomes/integrins/Shc/signaling/tyrosine phosphorylation

Introduction

Basement membranes provide cells with positional cues which can affect their proliferation and differentiation (Adams and Watt, 1993). It is now clear that cell-matrix interactions are in large part mediated by integrins (Ruoslahti, 1991; Hynes, 1992) and that ligation of integrins results in intracellular signaling (Juliano and Haskill, 1993; Giancotti and Mainiero, 1994). Many of the influences of basement membranes on cellular behavior can be recapitulated *in vitro* by laminins or blocked with anti-laminin antibodies (Adams and Watt, 1993). It is therefore important to elucidate the mechanisms by which

binding of laminins to integrins results in the activation of signal transduction pathways.

Laminins are a growing family of obligatory components of basement membranes expressed in a tissue- and development-specific manner (Engvall, 1993). At least six cell surface receptors, including various β_1 integrins and the $\alpha_6\beta_4$ integrin, have been implicated in binding to laminins and in many cases their binding specificities appear to overlap (Mercurio, 1990; Hynes, 1992). Cell adhesion to laminins, however, results in different patterns of gene expression depending on cell type and perhaps developmental stage (Di Persio *et al.*, 1991; Roskelley *et al.*, 1994), suggesting that specific signals may result from the engagement of distinct laminin binding integrins in different cells.

Focal adhesion kinase (FAK) (Shaller *et al.*, 1992) has been implicated in signaling from β_1 and β_3 integrins (Guan and Shalloway, 1992; Hanks *et al.*, 1992; Lipfert *et al.*, 1992). There is evidence suggesting that FAK can link integrins to the ras signaling pathway (Schlaepfer *et al.*, 1994), as well as induce intracellular changes which are potentially important for assembly of the actin cytoskeleton, such as phosphorylation of paxillin and tensin (Burridge *et al.*, 1992; Bockholt and Burridge, 1993) and activation of Rho (McNamee *et al.*, 1992; Chong *et al.*, 1994). However, the mechanisms by which β_1 and β_3 integrins activate FAK have remained elusive so far. In particular, since ligation of the platelet integrin $\alpha_{IIb}\beta_3$ causes a cascade of tyrosine phosphorylation events prior to activation of FAK (Huang *et al.*, 1993) and since the latter event requires an additional co-stimulus provided by an agonist receptor (Shattil *et al.*, 1994), it is possible that FAK does not lie immediately downstream of the integrins. Thus although these results establish the role of integrins in signaling, they do not clarify how laminin-derived signals are transduced at the plasma membrane and how specificity of signaling is achieved.

The $\alpha_6\beta_4$ integrin is a receptor for various laminins and binds with the highest relative affinity to laminins 4 and 5 (Spinardi *et al.*, 1995). The highest levels of expression of $\alpha_6\beta_4$ are observed in the basal cell layer of stratified epithelia (Kajiji *et al.*, 1989), at the ends of endothelial sprouts during angiogenesis (Enenstein and Kramer, 1994), in Schwann cells at the onset of myelination (Einheber *et al.*, 1993) and in CD4⁻CD8⁻ pre-T lymphocytes entering the thymus (Wadsworth *et al.*, 1992), suggesting the involvement of $\alpha_6\beta_4$ in various morphogenetic events. In addition, increased levels of $\alpha_6\beta_4$ are expressed in squamous, but not basal, carcinomas in humans (Kimmel and Carey, 1986; Savoia *et al.*, 1993) and suprabasal expression of $\alpha_6\beta_4$ is associated with malignant progression during mouse skin carcinogenesis (Tennenbaum *et al.*, 1993). Elucidation of the signal transduction mechanism of the $\alpha_6\beta_4$ integrin may, therefore, help us to understand

the differential effects induced by basement membranes in different normal cell types, as well as the significance of $\alpha_6\beta_4$ up-regulation in cancer cells.

The cytoplasmic domain of β_4 may provide the $\alpha_6\beta_4$ integrin with unique cytoskeletal and signaling interactions. The β_4 tail is very large (~1000 amino acids) and bears no homology with the short cytoplasmic domains of other known β subunits, including the β_1 and β_3 integrins, which are known to activate FAK. It contains, toward its C-terminus, two pairs of type III fibronectin (Fn)-like modules interrupted by a 142 amino acid long sequence (Connecting Segment) (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990). Furthermore, in contrast to the β_1 and β_3 integrins, which localize to focal adhesions, the $\alpha_6\beta_4$ integrin is found concentrated in hemidesmosomes (Carter *et al.*, 1990; Stepp *et al.*, 1990). Recent results demonstrate that $\alpha_6\beta_4$ plays a necessary role in the assembly of hemidesmosomes (Spinardi *et al.*, 1995). Upon binding to extracellular ligand, $\alpha_6\beta_4$ associates with cytoskeletal elements of hemidesmosomes, thereby linking the basement membrane to the keratin filament system. This $\alpha_6\beta_4$ function requires a specific region of the unique β_4 cytoplasmic domain, comprising the first pair of type III Fn-like repeats and the Connecting Segment (Spinardi *et al.*, 1993). Collectively, the unique structure, subcellular localization and cytoskeletal interactions of $\alpha_6\beta_4$ suggest that it may transduce intracellular signals by mechanisms distinct from those used by other integrins.

We here provide evidence that signal transduction by the $\alpha_6\beta_4$ integrin is mediated by an associated tyrosine kinase capable of phosphorylating the β_4 subunit. Mutations at a tyrosine activation motif (TAM) in the β_4 tail prevented the incorporation of $\alpha_6\beta_4$ into hemidesmosomes, but not the binding of Shc and Grb2, indicating that these two functions are mediated by phosphorylation of distinct integrin motifs.

Results

Ligation of the $\alpha_6\beta_4$ integrin induces tyrosine phosphorylation of the β_4 subunit

To examine the role of tyrosine phosphorylation in signal transduction by the $\alpha_6\beta_4$ integrin we asked if ligation of the extracellular portion of the integrin resulted in tyrosine phosphorylation of its component α or β subunits. To obtain selective ligation of $\alpha_6\beta_4$ in the absence of any concomitant stimulation caused by growth factors or cell shape changes, human epidermoid carcinoma A431 cells were serum starved, detached and then incubated in suspension with polystyrene beads coated with the anti- β_4 monoclonal antibody 3E1 or the control anti-MHC monoclonal antibody W6.32. Tyrosine phosphorylation of $\alpha_6\beta_4$ was monitored over time by immunoprecipitation with the 3E1 antibody followed by immunoblotting with anti-phosphotyrosine (P-Tyr) antibodies. As shown in Figure 1, the β_4 subunit was transiently phosphorylated on tyrosine in cells treated with anti- β_4 beads, but was not significantly phosphorylated in cells treated with control beads. In addition, no tyrosine phosphorylation of β_4 was observed in cells incubated with soluble 3E1 antibodies (not shown). These observations indicate that antibody-mediated cross-linking of $\alpha_6\beta_4$ results in activation

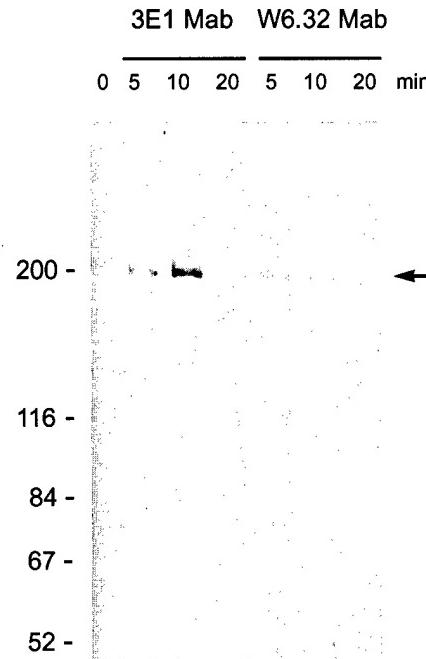


Fig. 1. Ligation of the extracellular portion of $\alpha_6\beta_4$ induces tyrosine phosphorylation of β_4 . Serum-starved A431 cells were stimulated in suspension for the indicated times with beads coated either with the anti- β_4 monoclonal antibody 3E1 or the anti-MHC monoclonal antibody W6.32. Equal amounts of total proteins were immunoprecipitated with the 3E1 antibody and probed by immunoblotting with polyclonal anti-P-Tyr antibodies. Molecular weight markers are indicated in kDa.

of a tyrosine kinase capable of phosphorylating the β_4 subunit.

To test whether the $\alpha_6\beta_4$ integrin is associated with cytoplasmic protein kinase(s), immune complex kinase assays were performed. The A431 cells, which express several β_1 integrins, as well as $\alpha_6\beta_4$, were immunoprecipitated with the monoclonal antibodies 3E1 and AIIB2, directed against the β_4 and the β_1 integrin subunits respectively. The immunoprecipitated samples were subjected to kinase assay and analyzed by SDS-PAGE. As shown in Figure 2A, incubation of the anti- β_4 immunoprecipitate with [γ -³²P]ATP resulted in significant phosphorylation of a 200 kDa protein corresponding to β_4 , as well as lower level phosphorylation of an additional 140 kDa protein. Occasionally, additional proteins with apparent molecular masses of 50–70 kDa also underwent specific phosphorylation in the *in vitro* reaction. In contrast, despite the presence of a tyrosine phosphorylation consensus site in the cytoplasmic domain of the β_1 subunit (Tamkun *et al.*, 1986), incubation of the anti- β_1 immunoprecipitate with [γ -³²P]ATP did not yield any specific phosphorylation product under these experimental conditions. Similar results were obtained with Lovo human colon carcinoma and 804G rat bladder carcinoma cells, which both express endogenous $\alpha_6\beta_4$. In addition, analysis of 804G cells expressing either a wild-type or a tail-less human β_4 subunit from cDNA indicated that while the full-length subunit was efficiently phosphorylated in the immune complex kinase assay, the truncated protein was not (Figure 2A). These results indicate that the β_4 subunit is phosphorylated *in vitro* by a protein kinase(s) associated

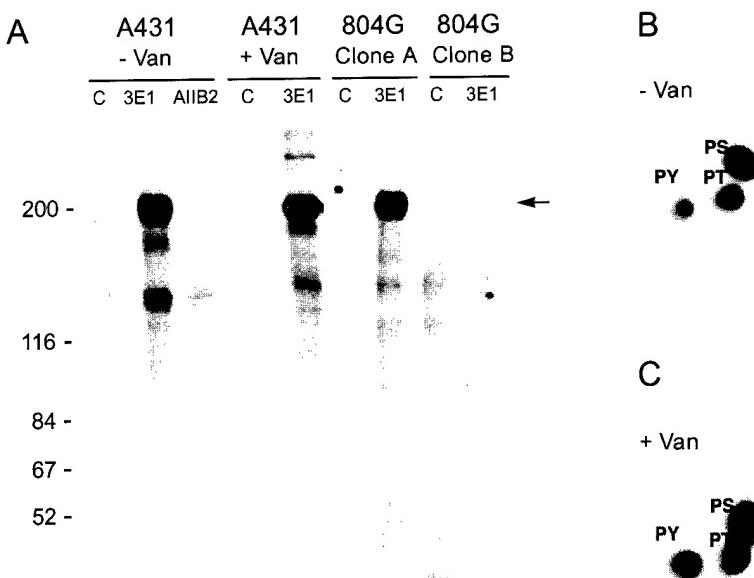


Fig. 2. The $\alpha_6\beta_4$ integrin is associated with a protein kinase(s) which can phosphorylate the β_4 subunit *in vitro*. (A) Phosphorylation of β_4 in an immune complex kinase assay. A431 cells were either directly extracted with Brij 96 buffer or treated with 10 μ M vanadate for 10 min prior to extraction. Rat 804G cells expressing a recombinant wild-type (clone A) or tail-less human β_4 subunit (clone B) were directly lysed with Brij 96 buffer. Equal amounts of total proteins were immunoprecipitated with control rabbit anti-mouse IgGs (C), anti- β_4 monoclonal antibody 3E1 (3E1) or anti- β_1 monoclonal antibody AIIB2 (AIIB2). The samples were subjected to an *in vitro* kinase assay and separated by SDS-PAGE. The arrow points to β_4 . (B) Phosphoamino acid analysis of *in vitro* labeled β_4 from untreated A431 cells (-Van). (C) Phosphoamino acid analysis of *in vitro* labeled β_4 from vanadate-treated A431 cells (+Van). Identical amounts of radioactivity were loaded in (B) and (C).

with the integrin and that this event requires an intact β_4 cytoplasmic domain.

Phosphoamino acid analysis indicated that the *in vitro* phosphorylated β_4 subunit contained a significant amount of phosphotyrosine, in addition to phosphothreonine and phosphoserine (Figure 2B). The incorporation of phosphate on tyrosine, threonine and serine residues was reduced, but not suppressed, if the immunoprecipitate was washed repeatedly under stringent conditions prior to the reaction (see Materials and methods), suggesting that the phosphorylation of β_4 was specific and was not caused by kinases contaminating the immunoprecipitate. Since the intracellular portion of $\alpha_6\beta_4$ does not contain a protein kinase domain, these results suggest that the integrin is physically associated with protein kinase(s) capable of phosphorylating β_4 on tyrosine, threonine and serine residues *in vitro*. Although it is likely that $\alpha_6\beta_4$ is associated with two protein kinases with distinct amino acid selectivity, these results do not exclude the possibility of an association with a dual specificity kinase.

Pretreatment of the cells with 10 μ M vanadate prior to detergent extraction and immune complex kinase assay enhanced the amount of phosphotyrosine recovered from β_4 after the reaction (compare Figure 2B and C), suggesting that tyrosine phosphorylation of β_4 is subject to negative regulation by protein tyrosine phosphatases. The relatively rapid time course of β_4 dephosphorylation observed after antibody-mediated engagement of the integrin (Figure 1) and the ability of micromolar concentrations of sodium orthovanadate to induce significant tyrosine phosphorylation of β_4 in intact cells (see Figure 7A and B, below) are also consistent with this hypothesis. Ligation of $\alpha_6\beta_4$ with anti- β_4 -coated beads prior to extraction and immune complex kinase assay only led to a modest increase in the amount of phosphotyrosine recovered from β_4 after the

reaction, suggesting that the association of $\alpha_6\beta_4$ with a tyrosine kinase is constitutive (data not shown). These results suggest that the antibodies to $\alpha_6\beta_4$ elicit tyrosine phosphorylation of the β_4 subunit *in vivo* by inducing dimerization or oligomerization of the integrin on the plasma membrane and thereby activating an associated protein tyrosine kinase and/or bringing it into close proximity to its target sequences in β_4 .

Association of $\alpha_6\beta_4$ with Shc and Grb2

Since tyrosine phosphorylation regulates the recruitment of SH2 domain molecules to activated cell surface receptors, we examined the possible involvement of SH2 domain proteins in signaling by $\alpha_6\beta_4$. To test if the adaptor protein Shc formed a complex with tyrosine-phosphorylated $\alpha_6\beta_4$, A431 cells were stimulated with anti- β_4 or anti-MHC beads and the resulting extracts were either immunoprecipitated with anti- β_4 antibodies and probed by immunoblotting with anti-Shc antibodies or immunoprecipitated with anti-Shc antibodies and probed with anti- β_4 antibodies. The results showed that p52^{Shc} is co-immunoprecipitated with $\alpha_6\beta_4$ from cells incubated with anti- β_4 beads, but not from those treated with anti-MHC beads (Figure 3A). Although the other two Shc isoforms, p46^{Shc} and p66^{Shc}, are expressed at levels comparable with that of p52^{Shc} in A431 cells (Pellicci, 1992) and are recognized by the antibodies used in this study, only a very modest amount of p46^{Shc} and no p66^{Shc} was detected in association with $\alpha_6\beta_4$. In addition, in accordance with the observation that $\alpha_6\beta_4$ does not contain tyrosine phosphorylation sites conforming to the consensus for binding to the p85 subunit of phosphatidylinositol-3-hydroxyl kinase or phospholipase C- γ (Songyang *et al.*, 1993), we did not detect an association of these SH2 molecules with tyrosine-phosphorylated $\alpha_6\beta_4$. Taken

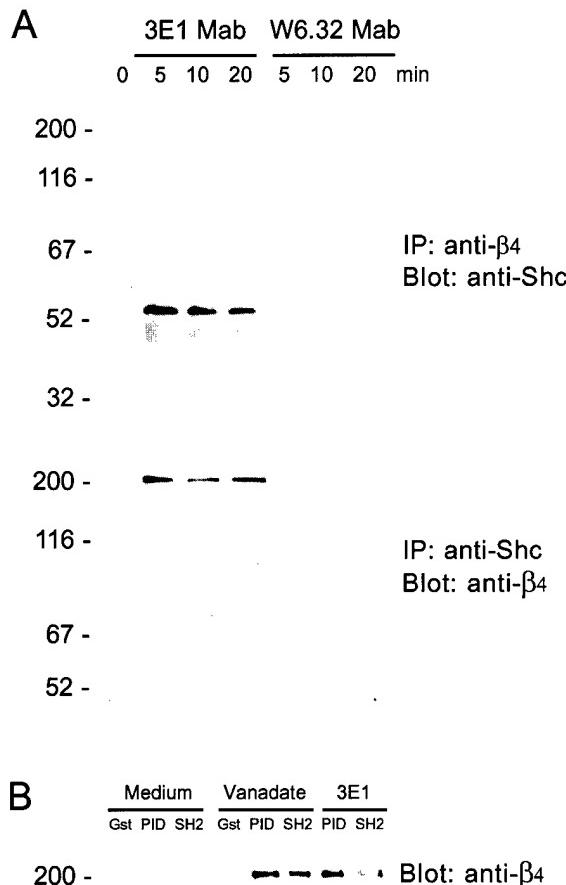


Fig. 3. Association of $\alpha_6\beta_4$ with Shc and Grb2. (A) Shc forms a complex with activated $\alpha_6\beta_4$ in intact cells. A431 cells were stimulated as indicated in Figure 1. Equal amounts of total proteins were immunoprecipitated with rabbit anti- β_4 peptide serum (top) or rabbit anti-Shc serum (bottom). The samples were probed by immunoblotting with anti-Shc monoclonal antibody (top) or anti- β_4 monoclonal antibody 450-11A (bottom). (B) Binding of the Shc PID and SH2 domains to β_4 . Rat 804G cells expressing the human wild-type β_4 subunit (clone A) were serum starved and treated with medium alone, 100 μ M sodium orthovanadate plus 3 mM H₂O₂ or stimulated in suspension with anti- β_4 beads for 10 min at 37°C. Denatured lysates were incubated with glutathione-agarose beads carrying the GST leader protein alone (GST) or GST-Shc PID domain (PID) or GST-Shc SH2 domain (SH2). Bound proteins were separated by SDS-PAGE and analyzed by immunoblotting with polyclonal anti- β_4 antibodies.

together, these results indicate that p52^{Shc} forms a specific complex with the activated $\alpha_6\beta_4$ integrin.

We next wondered if Shc could interact directly with the tyrosine-phosphorylated β_4 subunit and whether the interaction was mediated by the SH2 domain or the Phosphotyrosine Interaction Domain (PID) of Shc. 804G cells expressing a recombinant wild-type β_4 subunit were treated with sodium orthovanadate or incubated with anti- β_4 beads to induce β_4 phosphorylation. The extracts were denatured by heating in 1% SDS and incubated with agarose-immobilized GST fusion proteins encoding either the PID or the SH2 domain of Shc. Bound proteins were analyzed by immunoblotting with anti- β_4 antibodies. As shown in Figure 3B, both the PID and the SH2 domain of Shc bound to the β_4 subunit extracted from cells treated with vanadate or anti- β_4 antibodies, but not to β_4 from control, untreated cells. These results suggest that the

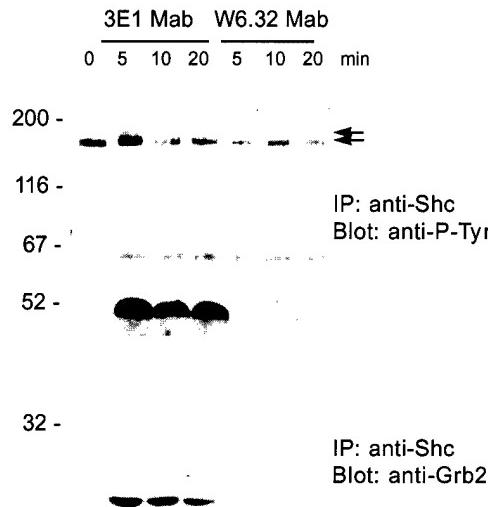


Fig. 4. Recruitment of Shc and Grb2 to the activated $\alpha_6\beta_4$ integrin. A431 cells were stimulated as indicated in Figure 1. Equal amounts of total proteins were immunoprecipitated with rabbit anti-Shc serum. The samples were probed by immunoblotting with a mixture of the two anti-P-Tyr monoclonal antibodies 4G10 and PY20 (top panel) or with monoclonal anti-Grb2 antibody (bottom panel).

tyrosine-phosphorylated β_4 subunit can interact directly with both the PID and the SH2 domain of Shc.

To examine the effect of $\alpha_6\beta_4$ ligation on tyrosine phosphorylation of Shc, A431 cells were incubated with anti- β_4 or control beads and immunoprecipitated with anti-Shc antibodies. The samples were analyzed by immunoblotting with anti-P-Tyr antibodies. As shown in Figure 4 (upper panel), treatment of the cells with anti- β_4 , but not control, beads led to tyrosine phosphorylation of p52^{Shc}. Two tyrosine-phosphorylated proteins were co-immunoprecipitated with Shc, a 195 kDa component which appeared to be constitutively associated with Shc and was not investigated further (lower arrow) and a 200 kDa molecule which was detected in association with Shc in cells treated with anti- β_4 , but not control, antibodies (upper arrow). Reprobing of the blot with the anti- β_4 monoclonal antibody 450-11A revealed that this latter protein corresponded to the tyrosine-phosphorylated β_4 subunit. These results indicate that upon forming a complex with activated $\alpha_6\beta_4$, p52^{Shc} becomes phosphorylated on tyrosine.

To examine the possibility that tyrosine-phosphorylated Shc associates with Grb2 upon ligation of $\alpha_6\beta_4$, extracts derived from A431 cells treated with anti- β_4 or control beads were subjected to immunoprecipitation with anti-Shc antibodies followed by immunoblotting with anti-Grb2 antibodies. The result indicated that Grb2 forms a complex with Shc in cells stimulated with anti- β_4 , but not control, beads (Figure 4, lower panel). Grb2 could also be detected in anti- β_4 immunoprecipitates from stimulated cells, but in lower amounts than in the anti-Shc immunoprecipitates. Together with the observation that the β_4 tail does not contain consensus Grb2 binding motifs (Songyang *et al.*, 1993), these results suggest that the association of Grb2 with $\alpha_6\beta_4$ is mediated by p52^{Shc} and contingent upon its tyrosine phosphorylation. Collectively these findings indicate that the two adaptors Shc and Grb2 interact sequentially with $\alpha_6\beta_4$, thereby potentially linking the integrin to the *ras* signaling pathway.

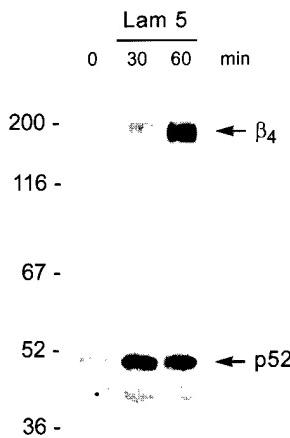


Fig. 5. Adhesion to laminin 5 results in tyrosine phosphorylation of the β_4 subunit and Shc. A431 cells were serum starved, detached with EDTA and either kept in suspension or plated on laminin 5 matrix-coated dishes for the indicated times. After extraction, the samples were immunoprecipitated with polyclonal antibodies to β_4 (top panel) or Shc (bottom panel). The samples were probed by immunoblotting with a mixture of the two anti-P-Tyr monoclonal antibodies 4G10 and PY20.

Cell adhesion to laminin 5 results in tyrosine phosphorylation of β_4 and p52^{Shc}

We next asked whether the above-described intracellular events also occurred in response to engagement of $\alpha_6\beta_4$ by extracellular matrix ligand. A431 cells were serum starved, detached and either kept in suspension or plated for different times on laminin 5 matrix-coated plates. Tyrosine phosphorylation of β_4 and p52^{Shc} was monitored by immunoprecipitation with specific antibodies followed by immunoblotting with anti-P-Tyr antibodies. As shown in Figure 5, cell adhesion to laminin 5 resulted in tyrosine phosphorylation of β_4 , p52^{Shc} and, to a minor extent, p46^{Shc}, but these events occurred with slower kinetics than in cells incubated in suspension with anti- β_4 beads. Presumably this is because ligation of integrins during cell adhesion to extracellular matrix ligand does not occur as rapidly and synchronously as during incubation with antibody-coated beads. These results suggest that the binding of extracellular matrix ligands to $\alpha_6\beta_4$ results in the same intracellular changes that are observed upon antibody-mediated ligation of the integrin.

Phosphorylation of a tyrosine-based activation motif (TAM) in the β_4 cytoplasmic domain

To assess the biological significance of β_4 phosphorylation, we sought to examine the tyrosine phosphorylation sites in β_4 . Preliminary studies using a combination of deletion mutagenesis and immunoblotting with anti-P-Tyr antibodies pointed to the presence of major tyrosine phosphorylation sites in the β_4 Connecting Segment (data not shown). Inspection of the amino acid sequence of the Connecting Segment revealed three potential tyrosine phosphorylation sites: Tyr1343, Tyr1422 and Tyr1440. We noted that the closely spaced Tyr1422 and Tyr1440 are embedded in very similar amino acid contexts. In particular, both residues are followed at position +3 by a leucine. Tandem tyrosine phosphorylation sites with a leucine at position +3 play a critical role in signal transduction by antigen receptors and are commonly referred to as TAMs

β_4 Integrin Subunit	R	D	Y	1422	N	S	L	T	R	S	E	H	S	H	S	T	T	L	P	R	D	Y	1440	S	T	L
h ζ 1	QL	Y	NE		L	N	L	G	R	R	E	E	-	-	-	-	-	-	Y	D	V	L				
h ζ 2	GL	Y	NE		L	Q	K	D	K	M	A	E	-	-	-	-	-	-	Y	S	E	L				
h ζ 3	GL	Y	QG		L	S	T	A	T	K	D	T	-	-	-	-	-	-	Y	D	A	L				
h CD3 γ	QL	Y	QP		L	K	D	R	E	D	D	Q	-	-	-	-	-	-	Y	S	H	L				
h CD3 ϵ	PD	Y	EP		I	R	K	G	O	R	D	L	-	-	-	-	-	-	Y	S	G	L				
h CD3 δ	QV	Y	QP		L	R	D	R	D	D	A	Q	-	-	-	-	-	-	Y	S	H	L				
h Fcy R II A	GG	Y	MT		L	N	P	R	A	P	T	T	DD	DD	K	N	I	-	Y	L	T	L				
r Fcc R γ	AV	Y	TG		L	N	T	R	N	Q	E	T	-	-	-	-	-	-	Y	E	T	L				
r Fcc R β	RL	Y	EE		L	H	V	Y	S	P	I	-	-	-	-	-	-	-	Y	S	A	L				
m Ig α	NL	Y	EG		L	N	L	D	D	C	S	M	-	-	-	-	-	-	Y	E	D	I				
m Ig β	HT	Y	EG		L	N	I	D	Q	T	A	T	-	-	-	-	-	-	Y	E	D	I				
BLV gp30	SD	Y	QA		L	L	P	S	A	P	E	I	-	-	-	-	-	-	Y	S	H	L				

Fig. 6. Alignment of tyrosine activation motifs (TAMs) within the cytoplasmic domains of the human integrin β_4 subunit, the human TCR ζ and CD3 chains, human FcyRIIA, the rat FccR β and γ chains, the mouse Ig α and β chains associated with IgM and IgD on B cells and the envelope glycoprotein (gp30) of bovine leukemia virus (BLV).

or antigen recognition activation motifs (ARAMs) (Weiss and Littman, 1994). Figure 6 shows an alignment of the β_4 TAM with the other previously identified TAMs, which include those present in the T cell receptor (TCR), B cell receptor (BCR), Fcc and Fcy receptors and the bovine leukemia virus gp30 glycoprotein.

To determine if the β_4 TAM sequence is phosphorylated *in vivo* and examine the physiological significance of this event, we generated and then introduced into 804G cells β_4 cDNAs carrying either individual phenylalanine substitutions at Tyr1343, Tyr1422 and Tyr1440 or a combined replacement of Tyr1422 and Tyr1440. Fluorescence activated cell sorting (FACS) analysis indicated that the cDNA encoded mutant subunits Y1343F, Y1422F, Y1440F and Y1422F/Y1440F were expressed at the cell surface at levels comparable with that of wild-type recombinant β_4 .

Wild-type β_4 and phenylalanine mutant subunits were examined by *in vivo* labeling and phosphopeptide mapping. Since antibody- or ligand-induced cross-linking of $\alpha_6\beta_4$ did not produce the high level tyrosine phosphorylation of β_4 required for mapping, tyrosine phosphorylation of β_4 was obtained by exposing the cells to vanadate. Preliminary experiments of [³²P]orthophosphate labeling and phosphoamino acid analysis revealed that the wild-type β_4 subunit is constitutively phosphorylated on serine residues *in vivo*, but becomes phosphorylated on tyrosine residues upon vanadate treatment (Figure 7A and B). *Staphylococcus* V8 protease digestion of wild-type β_4 from vanadate-treated cells yielded five major phosphopeptides (S1–S3, Y5 and Y6) and a number of minor phosphopeptides (Y1–Y4) (Figure 7, top panel). Phosphoamino acid analysis of individual phosphopeptides indicated that the major phosphopeptides S1–S3 contain exclusively radioactive phosphoserine. This observation is consistent with their presence in phosphopeptide maps of β_4 isolated from unstimulated cells. In contrast, the two major phosphopeptides Y5 and Y6, as well as the minor phosphopeptides Y1–Y4, which were only detected in stimulated cells, were found to contain exclusively phosphotyrosine. We concluded that β_4 is phosphorylated at multiple tyrosine residues *in vivo*.

We next examined the phosphopeptide maps of mutant

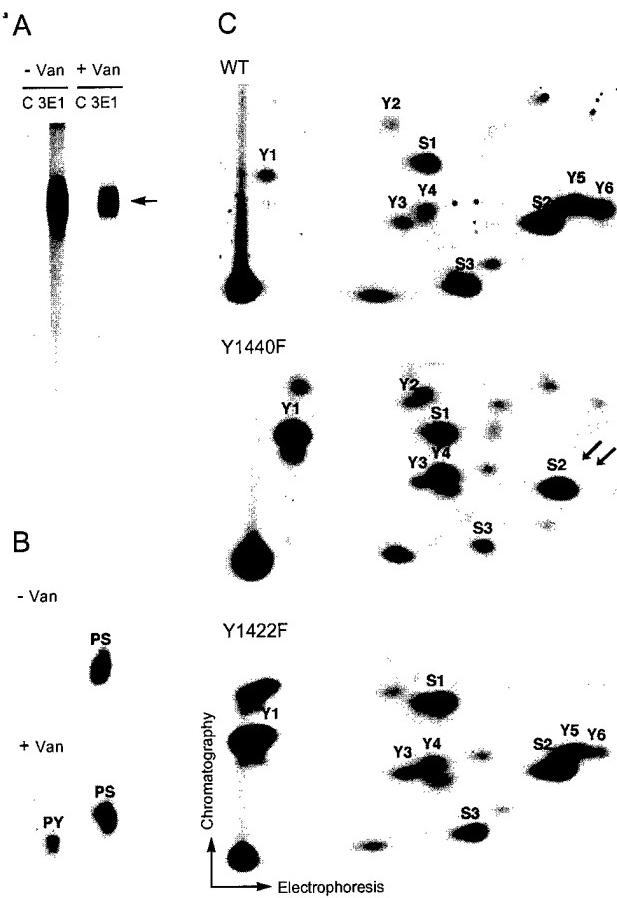


Fig. 7. *In vivo* phosphorylation of the β_4 TAM. (A) *In vivo* [32 P]orthophosphate labeling of β_4 . Rat 804G cells expressing human β_4 were labeled *in vivo* with [32 P]orthophosphate and then either left untreated (-Van) or treated with 500 μ M vanadate for 10 min (+Van). After extraction with RIPA buffer, the samples were immunoprecipitated with rabbit anti-mouse IgG (C) or the anti-human β_4 monoclonal antibody (3E1) and separated by SDS-PAGE. (B) Phosphoamino acid analysis of *in vivo* labeled β_4 . The 32 P-labeled β_4 bands of (A) were subjected to phosphoamino acid analysis. The top panel shows the phosphoamino acid analysis of *in vivo* labeled β_4 from untreated cells (-Van), the bottom panel that from vanadate treated cells (+Van). Identical amounts of radioactivity were loaded on the two TLC plates. (C) Mapping of β_4 tyrosine residues phosphorylated *in vivo*. Rat 804G cells expressing either the human wild-type β_4 subunit or the mutant subunits Y1422F or Y1440F were labeled *in vivo* with [32 P]orthophosphate, treated with 500 μ M vanadate for 10 min and immunoprecipitated with the anti-human β_4 antibody 3E1. After separation by SDS-PAGE, the radioactive bands corresponding to recombinant β_4 polypeptides were subjected to V8 protease digestion and the resulting phosphopeptides were separated by two-dimensional TLC. The top panel shows the map of wild-type β_4 (WT), the middle panel the map of mutant Y1440F (Y1440F) and the bottom panel the map of mutant Y1422F (Y1422F). Phosphoamino acid analysis indicated that the peptides S1-S3 contain exclusively phosphoserine and Y1-Y6 exclusively phosphotyrosine. Arrows point to the position of radioactive phosphopeptides affected by the Y1440F mutation.

subunits Y1422F and Y1440F. As shown in Figure 7C (middle panel), the replacement of Tyr1440 with phenylalanine caused the disappearance of peptides Y5 and Y6. The simultaneous disappearance of peptides Y5 and Y6 as a consequence of a single point mutation and their similar migration indicate that these peptides are closely related and that both contain Tyr1440. We also observed that the map derived from the Y1440F mutant subunit

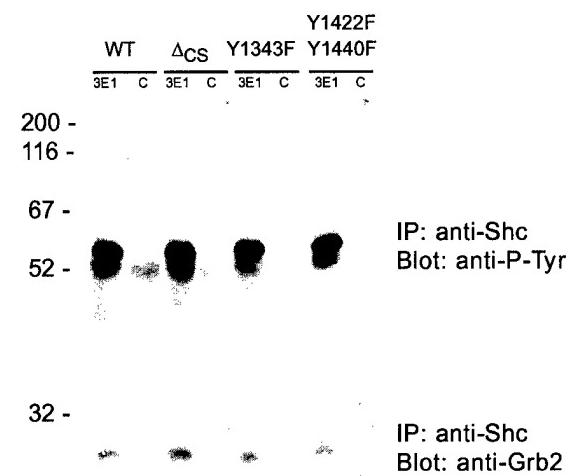


Fig. 8. Shc activation by recombinant β_4 subunits carrying mutations at the TAM sequence. Rat 804G cells expressing the wild-type human β_4 subunit (WT) or mutant versions lacking the entire Connecting Segment (Δ_{CS}) or carrying phenylalanine substitutions in the TAM (Y1422F/Y1440F) or outside the TAM (Y1343F) were incubated for 10 min with anti-human β_4 (3E1)- or anti-MHC (C)-coated beads and extracted. Equal amounts of total proteins were immunoprecipitated with rabbit anti-Shc serum. The samples were probed by immunoblotting with a mixture of the two anti-P-Tyr monoclonal antibodies 4G10 and PY20 (top panel) or with monoclonal anti-Grb2 antibody (bottom panel).

contained a number of novel peptides and that peptides Y1 and Y4 were more intensely radioactive than in wild-type β_4 . Presumably these events are a consequence of compensatory phosphorylation. The substitution of Tyr1422 with phenylalanine caused a reduction in the intensity of only a couple of phosphopeptides (Figure 7C, bottom panel). Also in this case we noticed compensatory phosphorylation (see, for example, peptide Y1). In contrast, the replacement of Tyr1343 with phenylalanine did not result in modification of any phosphopeptide (data not shown). We conclude that the β_4 tail is phosphorylated *in vivo* at multiple tyrosine residues: the C-terminal element of the TAM corresponds to one of the major sites of phosphorylation, while its N-terminal element may correspond to a minor one.

Activation of Shc by $\alpha_6\beta_4$ is not affected by mutations at the β_4 TAM

The role of the β_4 TAM in activation of the Shc/Grb2 pathway was examined using recombinant β_4 subunits carrying either a deletion of the Connecting Segment or phenylalanine substitutions in the β_4 TAM. Rat 804G cells expressing human wild-type or mutant β_4 subunits were incubated with beads coated with either the anti-human β_4 antibody 3E1 or the control anti-MHC antibody W6.32. The samples were immunoprecipitated with anti-Shc antibodies and probed with either anti-P-Tyr or anti-Grb2 antibodies. As shown in Figure 8 (top panel), β_4 subunits with a double mutation in the TAM, a single phenylalanine substitution outside the TAM but within the Connecting Segment or a complete deletion of the Connecting Segment mediated tyrosine phosphorylation of Shc as efficiently as wild-type β_4 . In all cases tyrosine phosphorylation of Shc resulted in recruitment of Grb2 (Figure 8, bottom panel).

Re-probing of the blot with the anti-human β_4 monoclonal antibody 450-9D indicated that all the mutant subunits had formed a specific complex with Shc upon stimulation with anti- β_4 beads (data not shown). These results indicate that the β_4 TAM sequence and the entire Connecting Segment are not required for linking $\alpha_6\beta_4$ to Shc and Grb2.

Phosphorylation of the β_4 TAM mediates association of the $\alpha_6\beta_4$ integrin with the cytoskeleton

Previous results indicated that association of the $\alpha_6\beta_4$ integrin with the cytoskeleton and consequent assembly of hemidesmosomes require a specific segment of the β_4 tail (Spinardi *et al.*, 1993, 1995). Since the β_4 TAM is part of this segment and selective inhibition of β_4 phosphorylation with the tyrosine kinase inhibitor herbimycin correlates with inhibition of hemidesmosome assembly (A.Pepe, F.Mainiero and F.G.Giancotti, unpublished results), we asked if phosphorylation of the β_4 TAM played a role in association of the integrin with the hemidesmosomal cytoskeleton. As the $\alpha_6\beta_4$ integrin incorporated in hemidesmosomes is largely resistant to extraction in non-ionic detergents (Spinardi *et al.*, 1993), we examined the Triton X-100 solubility of recombinant β_4 subunits carrying phenylalanine substitutions in the TAM. The result of this experiment indicated that the wild-type β_4 subunit and the control mutant subunit Y1343F, which carries a mutation outside the TAM, are associated predominantly with the Triton X-100-insoluble fraction. In contrast, the mutant subunit Y1422F was equally distributed in the detergent-soluble and -insoluble fractions and the mutant protein Y1440F was exclusively associated with the soluble fraction (Figure 9A). The mutant protein Y1422F/Y1440F was also recovered exclusively from the soluble fraction (data not shown). These results indicate that phosphorylation of the β_4 TAM is important for association of $\alpha_6\beta_4$ with the detergent-insoluble cytoskeleton.

We next examined the subcellular localization of the phenylalanine mutant β_4 subunits by immunofluorescence. Immunostaining with the 3E1 monoclonal antibody showed that wild-type human β_4 is in part diffusely distributed on the plasma membrane and in part concentrated at the basal cell surface within punctate, 'Swiss-cheese-like' structures corresponding to hemidesmosomes (Figure 9B, panel a; Spinardi *et al.*, 1993, 1995). In accordance with previous results, treatment with Triton X-100 prior to fixation eliminated the diffuse staining associated with the plasma membrane, but rendered more evident the 'Swiss-cheese-like' staining of hemidesmosomes (panel d). Cells expressing the control mutant subunit Y1343F, which carries a single phenylalanine substitution outside the connecting segment, displayed a staining pattern identical to that of control cells, indicating that this recombinant molecule is correctly targeted to hemidesmosomes (data not shown). In contrast, the staining pattern generated by the 3E1 antibody in cells expressing the mutant subunit Y1422F was mostly diffuse and associated with the plasma membrane (panel b). Although punctate staining could be detected in cells treated with Triton X-100 before fixation, this staining was much more scarce than that in control cells expressing wild-type β_4 and 'Swiss-cheese-like' structures were never

observed (panel e). This indicates that association of the mutant subunit Y1422F with hemidesmosomes is impaired as compared with that of wild-type β_4 . Finally, the 3E1 antibody generated only diffuse staining of the plasma membrane in cells expressing the mutant subunit Y1440F (panel c). Notably, virtually all staining was suppressed if the cells were treated with Triton X-100 prior to immunostaining (panel f). Identical results were obtained from an analysis of the subcellular localization of mutant subunit Y1422F/Y1440F (data not shown). Thus mutant β_4 subunits carrying either a single phenylalanine permutation at position 1440 or a double substitution at positions 1422 and 1440 can be detected at the cell surface, but not in hemidesmosomes. Taken together, these findings indicate that stable association of $\alpha_6\beta_4$ with the cytoskeleton at hemidesmosomes requires phosphorylation of both elements of the β_4 TAM.

Discussion

Although observations made in the past two decades point to a pivotal role of the extracellular matrix in controlling gene expression (Adams and Watt, 1993), the question of how integrins transduce signals at the plasma membrane level has remained in large part unsolved, despite intensive investigation. In this study we have examined the mechanism of signal transduction by the $\alpha_6\beta_4$ integrin. Our results indicate that ligand or antibody binding to $\alpha_6\beta_4$ causes tyrosine phosphorylation of the β_4 subunit and suggest that this event is mediated by a protein tyrosine kinase associated with the integrin. The results of phosphopeptide mapping and mutagenesis experiments indicate that the β_4 cytoplasmic domain is phosphorylated at multiple sites: one site, which corresponds to a bidentate TAM similar to those found in several immune receptors, mediates association of $\alpha_6\beta_4$ with the cytoskeleton of hemidesmosomes, while one or more distinct sites are involved in sequential recruitment of the adaptor molecules Shc and Grb2.

The mechanism of signaling by $\alpha_6\beta_4$ suggested by our results incorporates elements of other receptor systems, such as the recruitment of Shc and Grb2, as well as unique features, such as association with the hemidesmosomal cytoskeleton. Like many cytokine and immune receptors (Kishimoto *et al.*, 1994; Weiss and Littman, 1994), $\alpha_6\beta_4$ lacks an intracellular catalytic domain and relies on its association with a cytoplasmic tyrosine kinase for signal transduction. As tyrosine phosphorylation of β_4 can be triggered by adhesion to a laminin 5 matrix, as well as by antibody-mediated cross-linking, but not by soluble antibodies to $\alpha_6\beta_4$, it is likely that dimerization or oligomerization of the integrin is required either for activating the associated tyrosine kinase or for bringing it into close proximity to its target sequences in the β_4 tail. The identity of the tyrosine kinase associated with $\alpha_6\beta_4$ remains to be determined, but the selective ability of *src* family kinases to induce β_4 phosphorylation in co-transfection experiments (A.Curatola and F.G.Giancotti, unpublished results), together with previous observations indicating that the T cell and B cell receptor TAMs are phosphorylated by *src* family kinases (Weiss and Littman, 1994), suggest that $\alpha_6\beta_4$ may be associated with a *src* family member. The observation that the β_4 subunit can be phosphorylated on

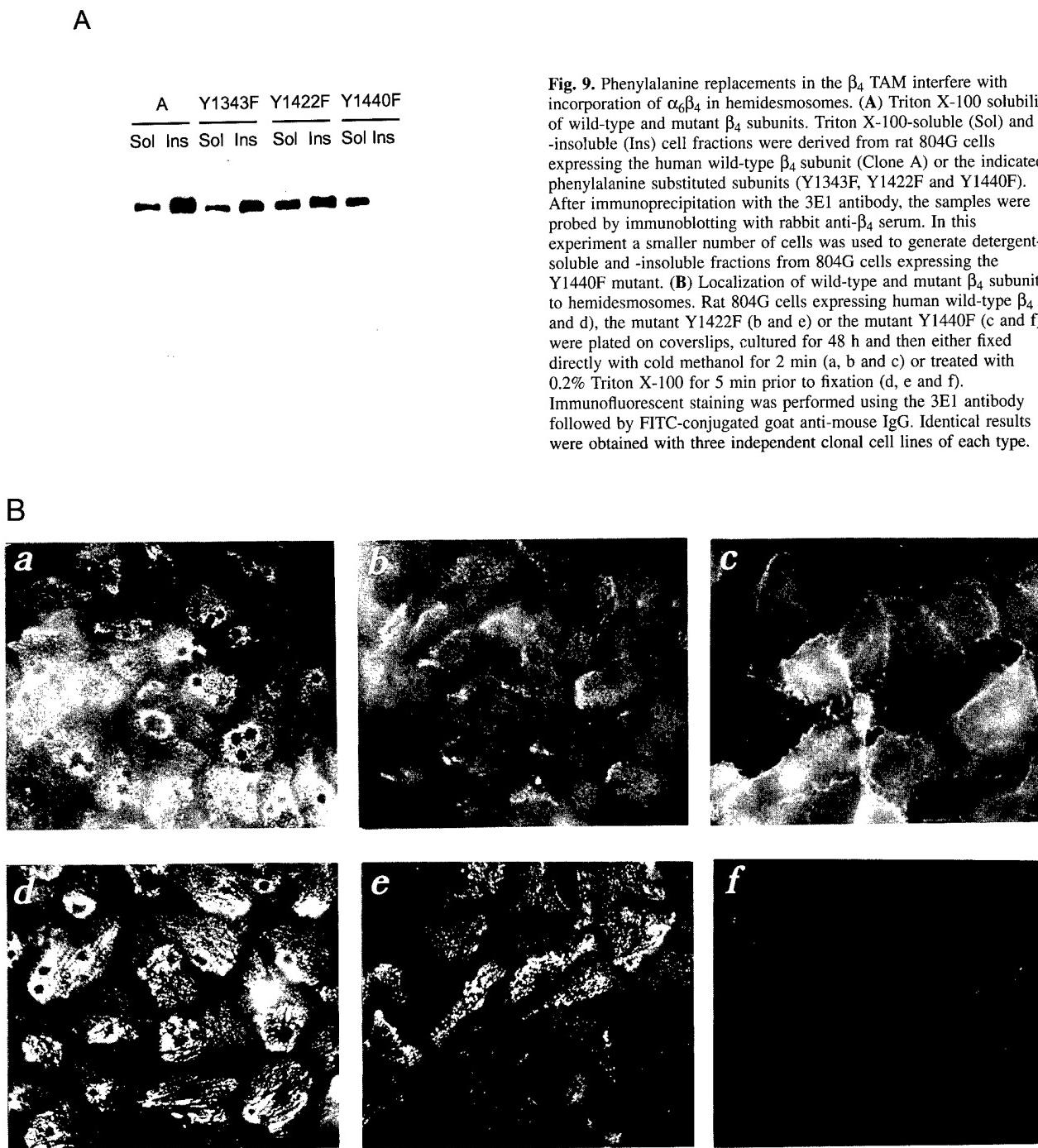


Fig. 9. Phenylalanine replacements in the β_4 TAM interfere with incorporation of $\alpha_6\beta_4$ in hemidesmosomes. (A) Triton X-100 solubility of wild-type and mutant β_4 subunits. Triton X-100-soluble (Sol) and -insoluble (Ins) cell fractions were derived from rat 804G cells expressing the human wild-type β_4 subunit (Clone A) or the indicated phenylalanine substituted subunits (Y1343F, Y1422F and Y1440F). After immunoprecipitation with the 3E1 antibody, the samples were probed by immunoblotting with rabbit anti- β_4 serum. In this experiment a smaller number of cells was used to generate detergent-soluble and -insoluble fractions from 804G cells expressing the Y1440F mutant. (B) Localization of wild-type and mutant β_4 subunits to hemidesmosomes. Rat 804G cells expressing human wild-type β_4 (a and d), the mutant Y1422F (b and e) or the mutant Y1440F (c and f) were plated on coverslips, cultured for 48 h and then either fixed directly with cold methanol for 2 min (a, b and c) or treated with 0.2% Triton X-100 for 5 min prior to fixation (d, e and f). Immunofluorescent staining was performed using the 3E1 antibody followed by FITC-conjugated goat anti-mouse IgG. Identical results were obtained with three independent clonal cell lines of each type.

serine and threonine residues in immune complex kinase assays indicates that $\alpha_6\beta_4$ may also be associated with other kinases, highlighting the complexity of $\alpha_6\beta_4$ function.

We have observed that ligation of $\alpha_6\beta_4$ results in its association with the adaptor protein Shc. This molecule contains two distinct domains capable of interacting with tyrosine-phosphorylated sequences: an N-terminal PID (Kavanaugh and Williams, 1994; Bork and Margolis, 1995) and a C-terminal SH2 domain (Pellicci *et al.*, 1992). The GST fusion protein binding experiments of this study suggest that both Shc domains can interact independently and directly with the tyrosine-phosphorylated β_4 subunit. Interestingly, the β_4 tail contains two tyrosine-based motifs

potentially able to interact with the Shc SH2 domain (Songyang *et al.*, 1994) and three N-X-X-Y motifs which could bind to the Shc PID (Kavanaugh *et al.*, 1995). Although definition of the β_4 sequences involved in interaction with Shc requires further mutagenesis experiments, the present results suggest that the PID and SH2 domains of Shc may bind to β_4 by a cooperative mechanism similar to that described for their binding to the epidermal growth factor receptor (Batzer *et al.*, 1995). As a consequence of its binding to $\alpha_6\beta_4$, Shc is phosphorylated on tyrosine, an event presumably mediated by the kinase associated with $\alpha_6\beta_4$, and then binds to Grb2. Several recent studies have indicated that Grb2 is stably associated

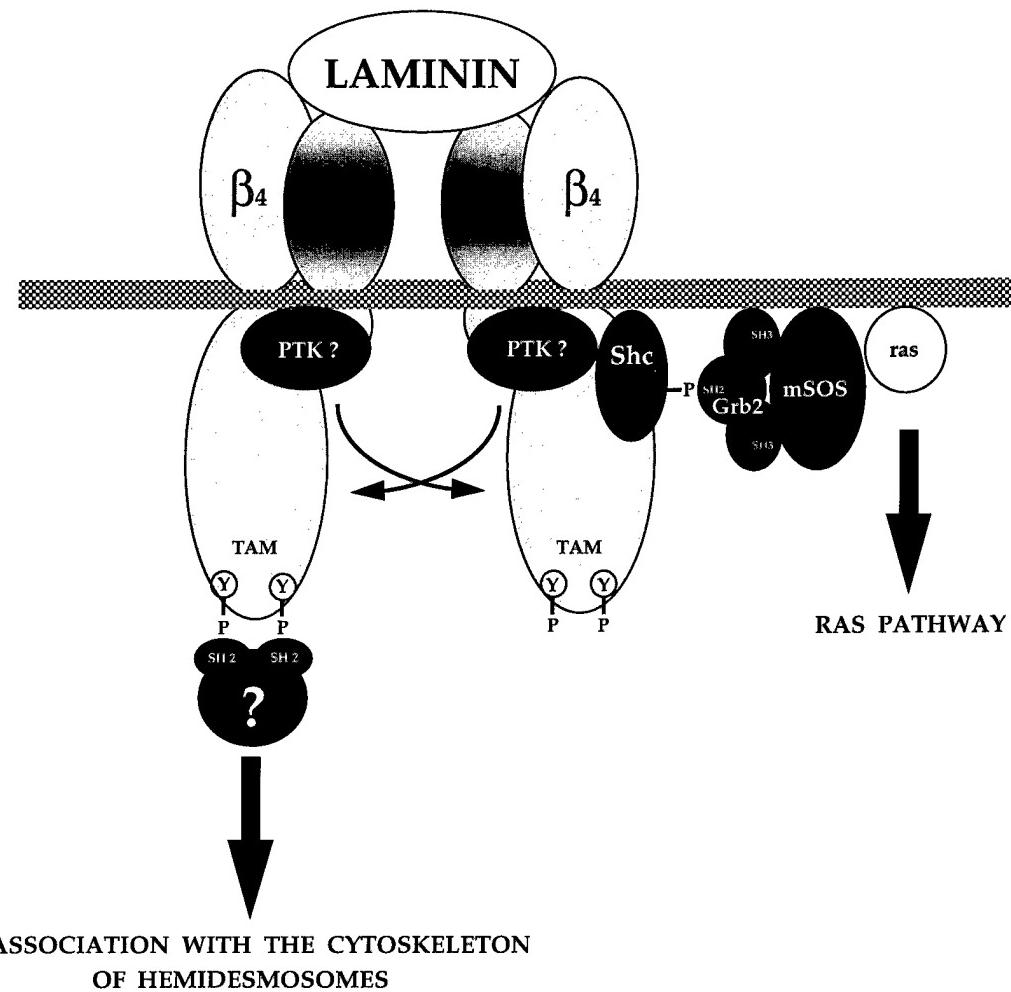


Fig. 10. Schematic model of $\alpha_6\beta_4$ integrin signal transduction. Laminin-induced dimerization or oligomerization of the integrin is followed by activation of an associated protein tyrosine kinase (PTK) that phosphorylates the β_4 tail at multiple residues. Phosphorylation at the β_4 TAM results in association of the integrin with the hemidesmosomal cytoskeleton. The identity of the SH2–SH2 signaling component that we hypothesize interacts with the phosphorylated β_4 TAM and mediates cytoskeletal association is unknown. Distinct β_4 tyrosine phosphorylation motifs mediate the recruitment of Shc. Subsequent tyrosine phosphorylation is likely to be mediated by the tyrosine kinase associated with the integrin. The Grb2–mSOS complex binds to tyrosine-phosphorylated Shc and is thereby recruited to the plasma membrane, where it can activate ras.

with the *ras* GTP exchanger mSOS (Schlessinger, 1994; Pawson, 1995). However, while in unactivated cells the complex is confined to the cytoplasm, in stimulated cells it is recruited to the activated receptors and therefore translocated to the plasma membrane, where it can activate *ras*. Our results therefore describe a molecular mechanism potentially linking the $\alpha_6\beta_4$ integrin to the *ras* signaling pathway. In the future it will be important to delineate the specific intracellular pathways activated by recruitment of Shc and Grb2 to $\alpha_6\beta_4$ and elucidate their effects on cell function.

Binding of laminin 5 to $\alpha_6\beta_4$ integrin plays an essential role in the organization of hemidesmosomes (Spinardi *et al.*, 1995). The results of this study suggest that this function requires phosphorylation of the β_4 TAM. Mutations which prevented tyrosine phosphorylation of the β_4 TAM also suppressed association of $\alpha_6\beta_4$ with hemidesmosomes. Interestingly, the replacement of Tyr1440 had a more drastic effect on $\alpha_6\beta_4$ function than mutation of Tyr1422, indicating that phosphorylation of the C-terminal tyrosine may be sufficient for partial functioning of the β_4 TAM. It must be noted that tyrosine

phosphorylation of β_4 occurs only transiently in response to ligation of $\alpha_6\beta_4$. In fact, virtually no tyrosine phosphorylated β_4 is detected in stably adherent cells, in which the majority of $\alpha_6\beta_4$ is in hemidesmosomes. Thus it is unlikely that the formation of hemidesmosomes depends on a stable interaction mediated by tyrosine-phosphorylated β_4 TAM. Instead, it is possible that the β_4 TAM is primarily involved in transducing a signal required for hemidesmosome assembly.

What is the nature of this signal? The TAM was originally identified as a common motif present in several immune receptors (Reth, 1989). In the TCR system, as a result of simultaneous binding of the TCR α/β heterodimer and co-receptor CD4 to the peptide-bearing MHC molecule, *lck* comes into close proximity to and phosphorylates the TAMs present in the multichain invariant CD3 complex. Phosphorylation of ζ chain TAMs provides a template for binding of the tyrosine kinase ZAP70 involved in subsequent downstream signaling events (Weiss and Littman, 1994). It is possible that the mechanism by which phosphorylation of the β_4 TAM regulates cytoskeletal assembly also involves binding to an SH2

domain-containing protein. The tyrosine kinases ZAP70 and syk contain two tandem SH2 domains through which they bind to the phosphorylated TAMs of the T cell and B cell receptors respectively (Weiss and Littman, 1994). These molecules, however, are restricted to the immune system. In addition, the spacing between Tyr1422 and Tyr1440 in β_4 is larger than the distance between the tyrosines in other TAMs. These observations raise the possibility that the β_4 TAM has a distinct binding specificity. To prove this model it will be necessary to identify the protein kinase or adaptor interacting with the β_4 TAM.

In sum, the results of this study suggest a model of signal transduction by $\alpha_6\beta_4$ integrin that involves a number of sequential steps (Figure 10). We hypothesize that upon binding to a multivalent extracellular matrix ligand $\alpha_6\beta_4$ dimerizes or oligomerizes on the plasma membrane, thereby activating an associated intracellular tyrosine kinase and/or juxtaposing it to its target sequences in the β_4 tail. The phosphorylated β_4 subunit then interacts with Shc and Grb2, as well as with molecules involved in assembly of hemidesmosomes. These two functions appear to be mediated by distinct motifs, because mutations in the β_4 TAM selectively interfere with association of the integrin with the hemidesmosomal cytoskeleton.

The $\alpha_6\beta_4$ signaling mechanism proposed here appears to be especially suited to allow fine tuning of distinct intracellular functions in response to diverse environmental cues. The level of phosphorylation of distinct receptor sites may diverge substantially depending on the nature of the extracellular ligand (Sloan-Lancaster *et al.*, 1994). Thus it is possible that the β_4 TAM and the distinct site involved in binding to Shc are differentially phosphorylated depending on the specific laminin isoform encountered by the cell or its oligomerization state. In addition, the level of phosphorylation of each site may vary with the cell type and its state of differentiation. This potential mechanism is attractive because it would allow a differential regulation of the *ras* pathway and assembly of hemidesmosomes depending on the matrix and cellular context. It is possible that the growth advantage of squamous carcinoma cells is at least in part related to overexpression of $\alpha_6\beta_4$ in these cells (Kimmel and Carey, 1986; Savoia *et al.*, 1993; Tennenbaum *et al.*, 1993) and to its ability to link to the *ras* pathway. Squamous carcinoma cells, however, lack well-organized hemidesmosomes (Schenk, 1979), suggesting that the signals responsible for hemidesmosome assembly may be defective in these cells. Thus these cells may represent an extreme example of the divergent regulation of $\alpha_6\beta_4$ -mediated signals.

Finally, the signal transduction mechanism described in this paper provides a rational basis for the effects of $\alpha_6\beta_4$ on morphogenesis and tumor progression. Although it is likely that the intracellular signals elicited by laminin binding to $\alpha_6\beta_4$ are unique, future studies will undoubtedly reveal the extent of signaling overlap between various integrins. The recent observation that $\alpha_v\beta_3$ associates with insulin receptor substrate 1 in insulin-stimulated cells (Vuori and Ruoslahti, 1994) suggests that an additional level of complexity in integrin signaling may result from interaction between growth factor- and adhesion-dependent pathways. In this context, the results of this study represent a first step toward understanding the mechanisms of signal transduction by integrins.

Materials and methods

Antibodies

The monoclonal antibody 3E1, reacting with the extracellular portion of human β_4 , and the rabbit polyclonal antiserum to the C-terminal peptide of β_4 have been described previously (Giancotti *et al.*, 1992). The anti- β_4 monoclonal antibody 450-9D and 450-11A have also been previously characterized (Kennel *et al.*, 1990). The monoclonal antibody AIIIB2 binds to the extracellular portion of the human β_1 subunit (Werb *et al.*, 1989). The anti-MHC monoclonal antibody W6.32 reacts with human and cultured rat cells (Kahn-Perles *et al.*, 1987). The rabbit polyclonal anti-P-Tyr serum 72 was produced according to published procedures (Kamps and Sefton, 1988). The monoclonal anti-P-Tyr antibody 4G10 was from UBI (Lake Placid, NY). The monoclonal anti-P-Tyr antibody PY20 and the monoclonal anti-Shc antibody were from Transduction Laboratories (Lexington, KY). The polyclonal anti-Shc serum 410 was obtained by immunizing a rabbit with a GST fusion protein containing the SH2 domain of the protein (Batzler *et al.*, 1995). The monoclonal antibody EL-6 recognizes an epitope in the SH2 domain of Grb2.

Constructs and transfections

All eukaryotic expression constructs were assembled in the CMV promoter-based vector pRC-CMV (Invitrogen Corp., San Diego, CA). The plasmids encoding the wild-type and tail-less human β_4 subunits have been previously described (Spinardi *et al.*, 1993). To generate the construct pCMV- β_4 Δ1314–1486, which directs expression of a truncated β_4 subunit lacking the Connecting Segment (Δ_{CS}), we employed the polymerase chain reaction (PCR) to engineer a DNA fragment encoding β_4 residues 1315–1485 flanked by *SacI* (5'-end) and *NorI* (3'-end) sites. The 4.8 kb *HindIII*–*XbaI* fragment of β_4 was subcloned into pSL1180 (Pharmacia, Piscataway, NJ), thus generating pSL1180- β_4 , and the 5.2 kb *NorI*–*SacI* fragment of this plasmid was ligated to the PCR-generated β_4 fragment. The 4.3 kb *BspEI*–*XbaI* fragment of the resulting plasmid was finally ligated to the 6.3 kb *XbaI*–*BspEI* fragment of pCMV- β_4 . Phenylalanine substitutions were introduced into β_4 using the Altered Sites *in vitro* mutagenesis system (Promega, Madison, WI). Correctness of all the constructs was verified by sequencing. Rat bladder carcinoma 804G cells were transfected with the various expression constructs and pSV-neo as previously described (Giancotti *et al.*, 1994). Clones expressing comparable levels of each recombinant β_4 polypeptide were selected by FACS analysis. Immunoprecipitation of cells labeled metabolically with [³⁵S]methionine was used to verify correct assembly of the recombinant β_4 polypeptides with the endogenous α_6 subunit (Spinardi *et al.*, 1993).

GST fusion proteins encoding the murine Shc PID (residues 1–209) and SH2 domains were expressed and purified on glutathione–agarose beads as previously described (Blaikie *et al.*, 1994).

Biochemical methods

To obtain selective ligation of $\alpha_6\beta_4$ in the absence of any co-stimulus, the cells were serum starved, detached with 10 mM ethylenediamine tetraacetate (EDTA) and then resuspended at 20×10⁶/ml. Aliquots (200 µl) of this cell suspension were incubated at 37°C and either stimulated with 1.8×10⁸ polystyrene sulfate latex beads (2.5 µm diameter; IDC, Portland, OR) coated with the 3E1 or the control W6.32 monoclonal antibody (400 µg/ml) for the indicated times or left untreated. To obtain engagement of $\alpha_6\beta_4$ by a physiological ligand, the cells were serum starved, detached with EDTA and either kept in suspension or plated on laminin 5 matrix-coated dishes (Spinardi *et al.*, 1995) for the indicated times. At the end of the incubation the cells were extracted for 30 min at 0°C with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) or lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100) containing 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% aprotinin, 4 µg/ml pepstatin A, 10 µg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM EDTA and 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetate (EGTA) (all from Sigma, St Louis, MO).

To examine the detergent solubility of phenylalanine mutant β_4 subunits, subconfluent monolayers of the various clones were extracted on ice with 50 mM Tris, pH 7.5, 150 mM NaCl, 0.2% Triton X-100 and protease inhibitors for 5 min. The detergent-soluble fraction was recovered and the insoluble cytoskeletons were washed and then extracted with RIPA buffer and protease inhibitors. Detergent-soluble and -insoluble fractions derived from the same sample were directly compared.

Immunoprecipitation and immunoblotting were performed as previously described (Giancotti and Ruoslahti, 1990; Giancotti *et al.*, 1992). Nitrocellulose-bound antibodies were detected by chemiluminescence with ECL (Amersham Life Sciences, Little Chalfont, UK).

For binding studies, rat 804G cells expressing the human wild-type β_4 subunit were serum starved and treated with 100 μ M sodium orthovanadate plus 3 mM H₂O₂ or stimulated in suspension with anti- β_4 beads for 10 min at 37°C. After extraction in SDS buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% SDS) with protease inhibitors, the lysates were heated for 5 min at 80°C, sonicated and diluted with 9 vol. lysis buffer. Glutathione-agarose beads carrying the GST fusion proteins were incubated with the denatured lysates (10 μ g fusion protein/1 mg total proteins) for 2 h at 4°C, washed and boiled in SDS-PAGE sample buffer. Samples were separated by SDS-PAGE and analyzed by immunoblotting with polyclonal anti- β_4 antibodies.

For immune complex kinase assay, subconfluent cell monolayers were extracted with 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Brij 96 and phosphatase and protease inhibitors. After clarification, the extracts were immunoprecipitated as described above. The affinity beads were washed extensively with the extraction buffer without phosphatase inhibitors and then equilibrated in kinase buffer (10 mM Tris, pH 7.4, 10 mM MnCl₂, 20 mM *p*-nitrophenylphosphate). The kinase reaction was initiated by adding 50 μ l kinase buffer containing 20 μ Ci [γ -³²P]ATP (4500 Ci/mmol; ICN Biomedicals Inc., Irvine, CA) to the beads and continued at 30°C for 30 min. The reaction was stopped by boiling the samples for 5 min in SDS-PAGE sample buffer.

Phosphoamino acid analysis was performed as described by Boyle *et al.* (1991). ³²P-Labeled β_4 was eluted from fixed polyacrylamide gels and precipitated with 20% trichloroacetic acid. ³²P-Labeled peptides were scraped off TLC plates, eluted in pyridine and lyophilized. Both types of sample were subjected to acid hydrolysis in 6 N HCl at 110°C for 1 h. Phosphoamino acids were separated by two-dimensional TLC electrophoresis in pH 1.9 buffer (2.5% formic acid, 7.8% acetic acid) for the first dimension (1.5 kV, 40 min) and in pH 3.5 buffer (5% acetic acid, 0.5% pyridine) for the second dimension (1.5 kV, 30 min). Non-radioactive standards were detected by ninhydrin staining, while radiolabeled phosphoamino acids were observed by autoradiography.

Phosphopeptide mapping was performed essentially as described by Boyle *et al.* (1991). Cells were labeled metabolically with [³²P]orthophosphate (3 mCi/ml; ICN) for 3 h and then either treated with 500 μ M sodium orthovanadate and 3 mM H₂O₂ for 10 min at 37°C or left untreated. After immunoprecipitation with the 3E1 antibody, the samples were transferred to nitrocellulose. The nitrocellulose fragments containing β_4 were soaked in 0.5% polyvinylpyrrolidone (PVP-360; Sigma), 100 mM acetic acid at 37°C for 30 min. Complete digestion was achieved by incubating the bands in 200 μ l 50 mM phosphate buffer, pH 7.8, with 25 μ g *Staphylococcus aureus* V8 protease (Worthington Biochemical Corp., Freehold, NJ) for 48 h at 37°C. The samples were separated by two-dimensional TLC. Separation in the first dimension was achieved by electrophoresis in pH 1.9 buffer (1.5 kV, 50 min) and in the second by ascending chromatography in Phospho Chromatography buffer (37.5% *n*-butanol, 25% pyridine, 7.5% acetic acid).

Immunofluorescence

Cells were either fixed directly with cold methanol for 2 min or treated with phosphate-buffered saline containing 0.2% Triton X-100 for 5 min on ice prior to fixation with methanol. Immunostaining with 3E1 antibody was performed as previously described (Spinardi *et al.*, 1993, 1995). Secondary antibodies were species-specific. Samples were examined with a Zeiss Axiophot Fluorescent Microscope.

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The Intracellular Functions of $\alpha_6\beta_4$ Integrin Are Regulated by EGF

Fabrizio Mainiero, Angela Pepe, Mitchell Yeon, Yunling Ren, and Filippo G. Giancotti

Department of Pathology, Kaplan Cancer Center, New York University School of Medicine, New York 10016

Abstract. Upon ligand binding, the $\alpha_6\beta_4$ integrin becomes phosphorylated on tyrosine residues and combines sequentially with the adaptor molecules Shc and Grb2, linking to the *ras* pathway, and with cytoskeletal elements of hemidesmosomes. Since $\alpha_6\beta_4$ is expressed in a variety of tissues regulated by the EGF receptor (EGFR), we have examined the effects of EGF on the cytoskeletal and signaling functions of $\alpha_6\beta_4$. Experiments of immunoblotting with anti-phosphotyrosine antibodies and immunoprecipitation followed by phosphoamino acid analysis and phosphopeptide mapping showed that activation of the EGFR causes phosphorylation of the β_4 subunit at multiple tyrosine residues, and this event requires ligation of the integrin by laminins or specific antibodies. Immunoprecipitation exper-

iments indicated that stimulation with EGF does not result in association of $\alpha_6\beta_4$ with Shc. In contrast, EGF can partially suppress the recruitment of Shc to ligated $\alpha_6\beta_4$. Immunofluorescent analysis revealed that EGF treatment does not induce increased assembly of hemidesmosomes, but instead causes a deterioration of these adhesive structures. Finally, Boyden chamber assays indicated that exposure to EGF results in upregulation of $\alpha_6\beta_4$ -mediated cell migration toward laminins. We conclude that EGF-dependent signals suppress the association of activated $\alpha_6\beta_4$ with both signaling and cytoskeletal molecules, but upregulate $\alpha_6\beta_4$ -dependent cell migration. The changes in $\alpha_6\beta_4$ function induced by EGF may play a role during wound healing and tumorigenesis.

To fully understand embryonic development, tissue repair, and tumor invasion, it is important to elucidate the mechanisms by which growth factor- and integrin-dependent signals are integrated inside cells. It is known that integrins transmit positional cues from the extracellular matrix to the cell interior, and the mechanisms by which these signals affect cellular responses to growth and differentiation factors are being actively investigated (Juliano and Haskill, 1993; Giancotti and Mainiero, 1994; Schwartz et al., 1995). Conversely, growth factors and cytokines can modulate a number of integrin-dependent functions, including cell adhesion (Serve et al., 1995; Kinashi et al., 1995), cell migration (Chen et al., 1993; Matthay et al., 1993; Klemke et al., 1994), and cytoskeletal organization (Ridley and Hall, 1992; Ridley et al., 1992), but the mechanisms underlying these phenomena are less clear.

The interaction between growth factor receptors and integrins has been largely examined in fibroblasts and platelets. Most of the studies have focused on the focal adhesion kinase p125^{FAK} (Schaller et al., 1992). In addition to being activated and undergoing autophosphorylation in response to ligation of β_1 and β_3 integrins (Guan and Shal-

loway, 1992; Hanks et al., 1992; Lipfert et al., 1992), p125^{FAK} is the target of signals originating from a number of growth factors and mitogenic neuropeptides (Zachary and Rozengurt, 1992). The activation of p125^{FAK} has been linked to changes potentially important for the regulation of actin cytoskeleton, such as the phosphorylation of paxillin and tensin (Burridge et al., 1992; Bockholt and Burridge, 1993) and the activation of Rho (McNamee et al., 1992; Chong et al., 1994) and PI-3 kinase (Chen and Guan, 1994). In addition, activated p125^{FAK} can combine with the Grb2/mSOS complex potentially leading to stimulation of the *ras*-MAP (mitogen-activated protein) kinase pathway (Schlaepfer et al., 1994), and insulin stimulation promotes association of the $\alpha_v\beta_3$ integrin with the Insulin Receptor Substrate 1 and the Grb2/mSOS complex (Vuori and Ruoslahti, 1994). These observations suggest that integrin- and growth factor-dependent signals may converge on p125^{FAK} and Insulin Receptor Substrate 1 to regulate gene expression and the actin cytoskeleton.

Much less is known about the integration of growth factor- and integrin-dependent signals in epithelial and other cells that are in contact with the basement membrane. The $\alpha_6\beta_4$ integrin is expressed in epithelial, endothelial, and Schwann cells and binds to various isoforms of the basement membrane component laminin (Lee et al., 1992; Niessen et al., 1994; Spinardi et al., 1995). Our previous studies have focused on the mechanisms by which this integrin interacts with the cytoskeleton and with signaling molecules. In contrast to other integrins that localize to fo-

Address all correspondence to Filippo G. Giancotti, Department of Pathology, MSB 548, New York University School of Medicine, 550 First Avenue, New York, NY 10016. Tel.: (212) 263-5343. Fax: (212) 263-8211. e-mail: gianc01@mrcr6.med.nyu.edu.

Y. Ren's present address is 21-17 36th Street, Astoria, NY 11105.

cal adhesions or otherwise interact with the actin filament system, $\alpha_6\beta_4$ is found in hemidesmosomes in close proximity to molecules linking to the keratin filament system (Carter et al., 1990; Stepp et al., 1990). There is evidence indicating that the association of $\alpha_6\beta_4$ with the hemidesmosomal cytoskeleton requires the uniquely large cytoplasmic domain of β_4 and specifically a ~300-amino acid region, which includes the first two type III fibronectin-like modules and the connecting segment (Spinardi et al., 1993). The ability of a tail-less mutant β_4 subunit to produce a dominant negative effect on the assembly of hemidesmosomes without suppressing cell adhesion to laminins indicates that $\alpha_6\beta_4$ plays an essential role in organizing the hemidesmosomal cytoskeleton (Spinardi et al., 1995). Taken together, these observations suggest that laminin binding to $\alpha_6\beta_4$ promotes the nucleation of hemidesmosomal cytoskeleton, and this activity is mediated by the β_4 cytoplasmic domain.

Recent studies have indicated that ligation of the extracellular portion of $\alpha_6\beta_4$ causes tyrosine phosphorylation of the β_4 subunit, and this event is mediated by protein kinase(s) physically associated with the integrin. Coimmunoprecipitation experiments have shown that, upon ligation of the extracellular portion of $\alpha_6\beta_4$, the adaptor protein Shc forms a complex with the tyrosine-phosphorylated β_4 subunit. Shc is then phosphorylated on tyrosine residues and recruits the adaptor protein Grb2, thereby potentially linking $\alpha_6\beta_4$ to the *ras* pathway. The β_4 subunit is phosphorylated on multiple tyrosine residues *in vivo*, including a tyrosine-based activation motif (TAM)¹ resembling those found in the T cell and B cell receptors. Since phenylalanine substitutions at the β_4 TAM disrupt the association of $\alpha_6\beta_4$ with hemidesmosomes, but do not interfere with tyrosine phosphorylation of Shc and recruitment of Grb2, distinct sites in $\alpha_6\beta_4$ mediate assembly of the hemidesmosomal cytoskeleton and linkage to the *ras* pathway (Mainiero et al., 1995).

The $\alpha_6\beta_4$ integrin is expressed in a variety of epithelial tissues that are regulated by the EGF (Sonnenberg et al., 1990). In this study, we have examined the effects of EGF on the cytoskeletal and signaling functions of $\alpha_6\beta_4$. Our results indicate that activation of the EGF receptor (EGFR) causes tyrosine phosphorylation of the β_4 subunit, but this event is not followed by association of the integrin with Shc or by increased assembly of hemidesmosomes. In contrast, EGF-dependent signals interfere with the ability of activated $\alpha_6\beta_4$ to associate with both signaling and cytoskeletal molecules. Exposure to EGF causes deterioration of hemidesmosomes and leads to increased $\alpha_6\beta_4$ -mediated cell migration toward laminins.

Materials and Methods

Cell Lines, Transfections, Antibodies, and Extracellular Matrix Molecules

Human epidermoid carcinoma A431 cells were cultured in DME with 5% bovine FCS. Mouse mammary RAC-11P/SD cells (Sonnenberg et al., 1993) and rat bladder 804G cells (Izumi et al., 1981) were cultured in

1. Abbreviations used in this paper: BPAG 2, bullous pemphigoid antigen 2; EGFR, EGF receptor; MHC, major histocompatibility complex; TAM, tyrosine-based activation motif.

DME with 10% FCS or bovine calf serum, respectively. Human primary keratinocytes were cultured in keratinocyte growth medium (GIBCO BRL, Gaithersburg, MD). The 804G cells were cotransfected with the expression vector pRK5-hEGF-R, encoding a full-length human EGFR (Ullrich et al., 1984), and the hygromycin resistance plasmid pHBO by the calcium coprecipitation method (Giancotti et al., 1994). Stable cell lines expressing moderate levels of recombinant EGFR (25–35 times lower than the endogenous EGFR in A431 cells) were selected by fluorescence activated cell sorting analysis and cultured with medium supplemented with 200 μ g/ml hygromycin (Calbiochem-Novabiochem Corp., La Jolla, CA). NIH-3T3 cells overexpressing a recombinant human EGFR (clone HER 14) (Honegger et al., 1987) were cultured in DME supplemented with 10% bovine calf serum and genetin (GIBCO BRL).

The mAb 3E1 reacting with the extracellular portion of human β_4 and the rabbit polyclonal antiserum to the COOH-terminal peptide of β_4 were described previously (Giancotti et al., 1992). The mAbs BV7 and TS2/16 bind to the extracellular portion of the human β_1 subunit (Martin-Padura et al., 1994; Arroyo et al., 1992). The rabbit antiserum to the cytoplasmic domain of α_v was previously described (Vogel et al., 1993). The anti-major histocompatibility complex (MHC) mAb W6.32 reacts with human and cultured rat cells (Kahn-Perles et al., 1987). The rabbit polyclonal anti-P-Tyr serum #72 was produced according to published procedures (Kamps and Sefton, 1988). The monoclonal anti-P-Tyr antibody 4G10 was obtained from UBI (Lake Placid, NY). The monoclonal anti-P-Tyr antibody PY20 and the monoclonal anti-Shc antibody were from Transduction Laboratories (Lexington, KY). The polyclonal anti-Shc serum #54 was obtained by immunizing a rabbit with a glutathione-S-transferase fusion protein comprising the SH2 domain of the protein. The bullous pemphigoid antigen 2 (BPAG 2)-specific rabbit polyclonal antiserum was raised by immunization with a glutathione-S-transferase fusion protein comprising the major antigenic determinant of the mouse protein in the laboratory of Joulli Utto (Thomas Jefferson University, Philadelphia, PA). The antiserum against the $\alpha_6\beta_1$ integrin purified from human placenta was generated in the laboratory of Erkki Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA) as previously described (Argraves et al., 1986). This antiserum cross-reacts with rodent β_1 integrins and blocks their function. The agarose-coupled 1G2 mAb was purchased from Oncogene Science (Uniondale, NY).

Human plasma fibronectin and human placental laminin 4 were purchased from GIBCO BRL. Laminin 5 matrices were prepared as described previously (Sonnenberg et al., 1993; Spinardi et al., 1995).

Biochemical Methods

To test the effect of EGF on $\alpha_6\beta_4$, subconfluent A431 cells were serum starved and then treated with human recombinant EGF (Intergen Co., Purchase, NY). When indicated, the cells were detached by 10 mM EDTA and either kept in suspension or plated on dishes coated with extracellular matrix proteins before EGF stimulation. To examine the effect of selective ligation of $\alpha_6\beta_4$, the cells were plated on fibronectin-coated dishes, and then incubated with sulfate polystyrene latex beads coated with the 3E1 or control mAbs TS2/16 and W6.32. Stimulation of suspended cells with antibody-coated beads was performed as previously described (Mainiero et al., 1995). At the end of incubation, the cells were extracted for 30 min at 0°C with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycolate, 0.1% sodium dodecyl sulfate) or lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100) containing 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% aprotinin, 4 μ g/ml pepstatin A, 10 μ g/ml leupeptin, 1 mM PMSF, 1 mM EDTA, and 1 mM EGTA (all from Sigma Chemical Co., St. Louis, MO).

Immunoprecipitation and immunoblotting were performed as previously described (Giancotti and Ruoslahti, 1990; Giancotti et al., 1992). Nitrocellulose-bound antibodies were detected by chemiluminescence with ECL (Amersham Life Sciences, Little Chalfont, UK).

Phosphopeptide mapping was performed essentially as described by Boyle et al. (1991). Serum-starved cells were labeled metabolically with [³²P]orthophosphate (3 mCi/ml; ICN Biochemicals, Inc., Irvine, CA) for 3 h and then either treated with 250 ng/ml EGF for 5 min at 37°C or with 500 μ M sodium orthovanadate and 3 mM H₂O₂ for 10 min at 37°C. After immunoprecipitation with the 3E1 antibody, the samples were transferred to nitrocellulose. The nitrocellulose fragments containing β_4 were soaked in 0.5% polyvinylpyrrolidone (PVP-360; Sigma Chemical Co.), 100 mM acetic acid at 37°C for 30 min. Complete digestion was achieved by incubating the bands in 200 μ l of 50 mM phosphate buffer, pH 7.8, containing 25 μ g

of *Staphylococcus aureus* V8 protease (Worthington Biochemical Corp., Freehold, NJ) for 48 h at 37°C. The samples were separated by two-dimensional TLC. Separation in the first dimension was achieved by electrophoresis in pH 1.9 buffer (2.5% formic acid, 7.8% acetic acid) (1.5 kV, 50 min) and in the second, by ascending chromatography in phospho chromatography buffer (37.5% *n*-butanol, 25% pyridin, 7.5% acetic acid).

Phosphoamino acid analysis was performed as described by Boyle et al. (1991). 32 P-labeled β_4 was eluted from fixed polyacrylamide gels and precipitated with 20% TCA. 32 P-labeled peptides were scraped off TLC plates, eluted in 20% acetonitrile and 0.08% trifluoroacetic acid, and lyophilized. Both types of sample were subjected to acid hydrolysis in 6 N HCl at 110°C for 1 h. Phosphoamino acids were separated by two-dimensional TLC: electrophoresis in pH 1.9 buffer for the first dimension (1.5 kV, 40 min) and in pH 3.5 buffer (5% acetic acid, 0.5% pyridine) for the second dimension (1.5 kV, 30 min). Nonradioactive standards were detected by ninhydrin staining.

Adhesion and Migration Assays

Adhesion assays were performed essentially as previously described (Giancotti et al., 1985). Before the assay, the cells were serum starved and either treated with 100 ng/ml EGF for 5 min or left untreated. After detachment by incubation in 10 mM EDTA, they were washed and plated on extracellular matrix-coated plates in the presence of anti- β_1 serum at 1:50. The results were quantitated as previously described (Giancotti et al., 1986).

Cell migration assays were performed by using modified Boyden chambers containing porous (8- μ m) polycarbonate membranes (Nunc, Roskilde, Denmark). To measure migration toward fibronectin and laminin 4, the lower aspect of the membrane was coated with 10 μ g/ml of each extracellular matrix protein. To measure migration toward laminin 5, RAC-11P/SD cells were cultured on the lower aspect of the filter, and their laminin 5-containing matrix was prepared as described previously (Sonnenberg et al., 1993; Spinardi et al., 1995). Cells (50,000) were added to the upper chamber in 200 μ l of serum-free DME supplemented with 1% ITS+ (Collaborative Research, New Bedford, MA). EGF (50 ng/ml) and PDGF (5 ng/ml) were placed in the lower migration chamber in 500 μ l of the same medium. When indicated, the inhibitory anti- β_1 serum was added at 1:50 final dilution. After 12 or 48 h of incubation at 37°C, the cells that had migrated across the membrane were fixed with 3% paraformaldehyde, stained with crystal violet, and counted.

Immunofluorescence

The 804G transfectants and primary human keratinocytes were cultured on glass coverslips, starved for ~24 h and then treated with EGF, PDGF, or left untreated. After extraction with PBS containing 0.2% Triton X-100 for 5 min on ice, the cells were fixed with methanol and stained for 45 min with the various antibodies. The anti- β_4 cytoplasmic peptide rabbit serum was diluted 1:200. The anti-BPAG 2 IgGs were used at 25 μ g/ml, and the 3E1 mAb was used at 5 μ g/ml. After extensive washing, the cells were incubated for 45 min with 0.5–1 μ g/ml affinity-purified FITC-conjugated goat anti-rabbit or anti-mouse IgGs (Molecular Probes, Inc., Eugene, OR). The coverslips were mounted in Citi-Fluor (Chemical Laboratory of the University of Kent, Canterbury, UK). Samples were examined with a fluorescent microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY).

Results

EGF-mediated Tyrosine Phosphorylation of the β_4 Subunit

To test the hypothesis of a potential link between the intracellular responses elicited by EGF and the function of $\alpha_6\beta_4$ integrin, we examined if treatment with EGF could induce tyrosine phosphorylation of $\alpha_6\beta_4$ in cultured epithelial cells. The human epidermoid carcinoma A431 cells, which express high levels of the EGFR, were serum starved and either left untreated or exposed to EGF. Immunoprecipitation with the anti- β_4 mAb 3E1 followed by immunoblotting with anti-phosphotyrosine (anti-P-Tyr) antibodies indicated that treatment with EGF causes significant tyrosine phosphorylation of the β_4 subunit, sug-

gesting that $\alpha_6\beta_4$ is a direct or indirect target of the EGFR (Fig. 1 A). To explore the selectivity of the effect of EGF on β_4 phosphorylation, we asked if exposure to the growth factor also caused tyrosine phosphorylation of β_1 or α_v integrins. We reasoned that this experiment would have provided for a good control, as the cytoplasmic domains of β_1 , β_3 , β_5 , and β_6 contain a conserved sequence motif resembling a major tyrosine autophosphorylation site in the EGFR (Hynes, 1992). As shown in Fig. 1 A, immunoprecipitation with the anti- β_1 mAb BV7 or an anti- α_v cytoplasmic domain serum followed by immunoblotting with anti-P-Tyr antibodies showed that exposure to EGF does not induce tyrosine phosphorylation of β_1 or α_v containing integrins (Fig. 1 A). This result suggests that the effect of EGF on β_4 phosphorylation is selective. Experiments of [32 P]orthophosphate labeling and phosphoamino acid analysis were performed to confirm the ability of EGF to induce tyrosine phosphorylation of β_4 . The results indicated that the β_4 subunit is constitutively phosphorylated on serine, and it becomes phosphorylated on tyrosine residues in response to EGF treatment (Fig. 1 B). Taken together, these results demonstrate that the β_4 subunit is phosphorylated on tyrosine residues in cells exposed to EGF.

Immunoblotting with anti-phosphotyrosine antibodies indicated that the phosphorylation of β_4 induced by EGF is dose dependent. In A431 cells, we detected a significant level of β_4 phosphorylation in response to as little as 10 ng/ml EGF, and maximal phosphorylation in response to 250 ng/ml EGF (Fig. 2 A). The results of time course experiments indicated that the phosphorylation of β_4 induced by EGF in A431 cells follows a biphasic kinetics characterized by a first rapid peak occurring at 2 min and a second one at ~120 min from the initial challenge (Fig. 2 B). The decline in β_4 phosphorylation observed at 4 and 8 min after the initial stimulus may be related to the internalization of EGF receptor, a phenomenon that occurs rapidly after ligand binding (Beguinot et al., 1984). This interpretation is supported by the observation that the second peak of β_4 phosphorylation induced by EGF occurs at a time when the downregulation of the EGF receptor has already subsided (Teslenko et al., 1987). The stoichiometry of EGF-induced β_4 phosphorylation was estimated in A431 cells treated for 20 min with 50 ng/ml EGF. After extraction, the tyrosine-phosphorylated integrin was separated from the nonphosphorylated one by affinity chromatography on the anti-P-Tyr mAb 1G2, and both fractions were subjected to immunoblotting with anti- β_4 antibodies (not shown). Densitometric analysis of the results indicated that 83% of the total β_4 subunit had bound to the anti-phosphotyrosine affinity column. From these experiments, we concluded that the tyrosine phosphorylation of β_4 induced by EGF in A431 cells is rapid, dose dependent, and characterized by a high stoichiometry.

We next wondered if treatment with EGF caused tyrosine phosphorylation of β_4 also in normal epithelial cells, which express lower levels of the EGFR than A431 cells. As shown in Fig. 2 C (left), treatment of primary human keratinocytes with 10 ng/ml EGF caused significant tyrosine phosphorylation of the β_4 subunit. A similar result was obtained with rat epithelial 804G cells expressing moderate levels of recombinant human EGFR (35 times lower than the endogenous EGFR in A431 cells) (Fig. 2 C,

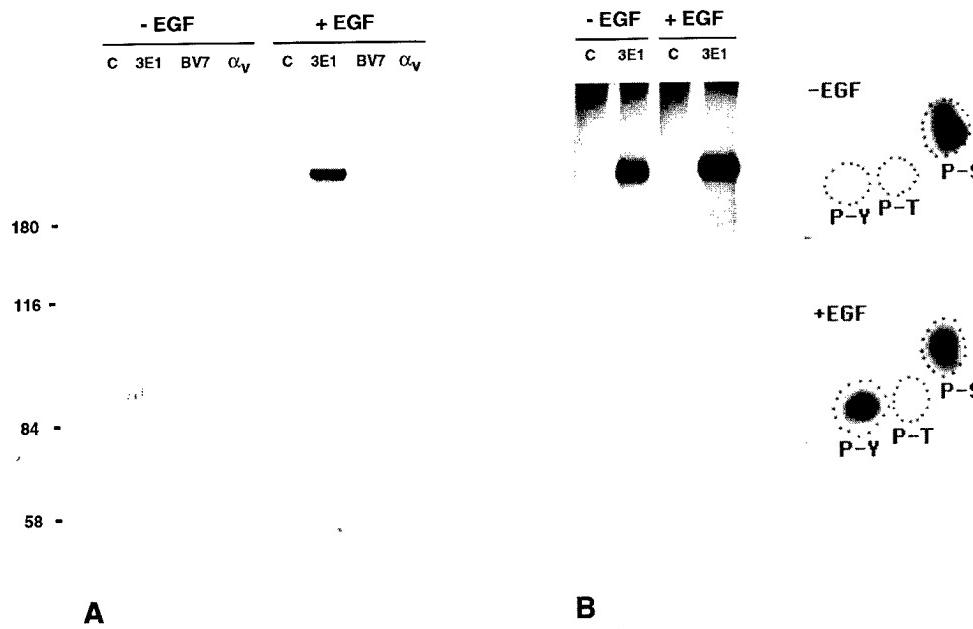


Figure 1. EGF-mediated tyrosine phosphorylation of the β_4 subunit. (A) A431 cells were serum starved and either left untreated or stimulated with 250 ng/ml EGF for 20 min. The samples were immunoprecipitated with control rabbit anti-mouse IgGs, anti-human β_4 mAb 3E1, anti-human β_1 mAb BV7, or anti- α_v cytoplasmic domain serum, and then probed by immunoblotting with polyclonal anti-P-Tyr antibodies. (B) A431 cells were serum starved, metabolically labeled with [³²P]orthophosphate, and then either left untreated or stimulated with 250 ng/ml EGF for 20 min. The samples were immunoprecipitated with control rabbit anti-mouse IgGs or anti-human β_4 mAb 3E1. The radioactive bands corresponding to β_4 were subjected to phosphoamino acid analysis.

right). These results indicate that EGF induces tyrosine phosphorylation of β_4 in cells that express moderate levels of EGFR, including primary epithelial cells.

To explore the mechanism by which the EGFR induces tyrosine phosphorylation of the β_4 subunit, we examined the ability of immunopurified EGFR to phosphorylate in vitro the $\alpha_6\beta_4$ integrin or fusion proteins reproducing the β_4 cytoplasmic domain. Despite undergoing significant autophosphorylation, the EGFR only weakly phosphorylated these potential substrates in in vitro assay (unpublished results). We also wondered if the EGFR and $\alpha_6\beta_4$ stably interacted in A431 cells, but coimmunoprecipitation experiments performed under mild detergent conditions failed to demonstrate a specific association of the two molecules (unpublished results). Although not conclusive, the results of these experiments are consistent with the hypothesis that in vivo the EGFR does not directly phosphorylate β_4 , but rather activates a signaling pathway that causes its phosphorylation.

EGF-mediated Tyrosine Phosphorylation of β_4 Requires Ligation of the Integrin by Extracellular Matrix Ligands or Antibodies

To examine if the ability of EGF to induce tyrosine phosphorylation of β_4 is influenced by cell adhesion, A431 cells were detached from the culture substratum, either kept in suspension or plated onto uncoated culture dishes for various times, and then treated with EGF. As shown in Fig. 3 A, in the absence of EGF treatment, no tyrosine phosphorylation of β_4 was detected in both suspended and stably adherent cells. Treatment with EGF did not result in tyrosine phosphorylation of β_4 in suspended cells. In con-

trast, the growth factor induced significant phosphorylation of β_4 in cells that had been plated on the culture dish for ≥ 4 h and were stably adherent. The ability of EGF to induce tyrosine phosphorylation of β_4 did not depend on cell-to-cell contact, as sparse and confluent cells were equally susceptible to the effect of the growth factor. Immunofluorescence experiments indicated that by 4 h of plating the $\alpha_6\beta_4$ integrin had already redistributed to the basal cell surface, presumably in response to extracellular matrix ligands deposited onto the culture substratum during adhesion (data not shown). These observations suggest a correlation between the recruitment of $\alpha_6\beta_4$ to the basal cell surface during adhesion and its susceptibility to EGF-mediated tyrosine phosphorylation.

To examine the hypothesis that the tyrosine phosphorylation of β_4 induced by EGF requires ligation of the integrin by extracellular matrix ligand, A431 cells were plated on dishes coated with the $\alpha_6\beta_4$ ligands laminin 5 and laminin 4 or the control ligand fibronectin, and then treated with EGF. Coating concentrations were adjusted so as to obtain the same extent of cell adhesion and spreading at 30 min. As shown in Fig. 3 B, plating of the cells on laminin 5 and 4 rendered the β_4 subunit fully susceptible to EGF-mediated phosphorylation. In contrast, plating on fibronectin had a more modest effect. The effect of EGF on β_4 phosphorylation was maximal during initial adhesion to laminins and then declined. In accordance with the observation that A431 cells adhere with a faster kinetics to laminin 5 than to laminin 4, the peak of β_4 phosphorylation occurred earlier on laminin 5 than on laminin 4. Plating A431 cells onto laminin 5 in the absence of EGF also induced tyrosine phosphorylation of β_4 , but this phosphorylation was of lower level and occurred with a slower kinet-

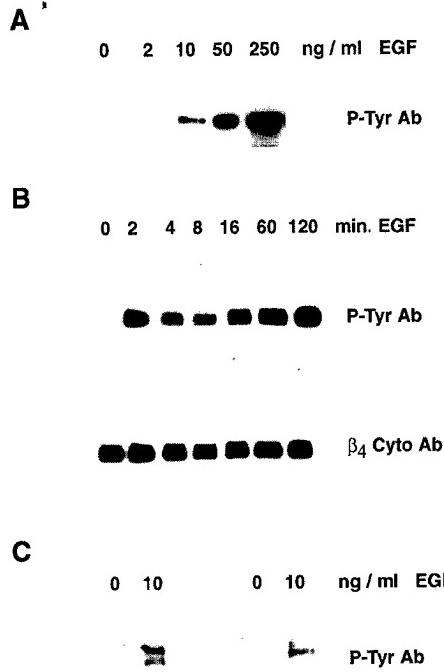


Figure 2. Dose dependence and kinetics of EGF-mediated tyrosine phosphorylation of β_4 . Serum-starved A431 cells were treated for 20 min with the indicated concentrations of EGF (*A*), or treated with 50 ng/ml EGF for the indicated times (*B*). After immunoprecipitation with the 3E1 mAb, the samples were probed by immunoblotting with polyclonal anti-P-Tyr or anti- β_4 cytoplasmic domain antibodies. Growth factor-starved primary human keratinocytes (*C, left*) and 804G cells expressing a recombinant EGFR (*C, right*) were treated with the indicated concentrations of EGF for 15 min. Immunoprecipitation was with anti- β_4 cytoplasmic domain antibodies and immunoblotting with polyclonal anti-P-Tyr antibodies.

ics than in the presence of EGF (Mainiero et al., 1995). Control experiments revealed that the ability of EGFR to undergo autophosphorylation, as well as to induce tyrosine phosphorylation of several cellular substrates, was similar in cells freshly plated on each one of the extracellular matrix proteins tested, including fibronectin (Fig. 3 *C*). These results are consistent with the notion that ligand binding to $\alpha_6\beta_4$ is required for optimal tyrosine phosphorylation of β_4 in response to EGF stimulation.

Since the A431 cells express at least another integrin, $\alpha_3\beta_1$, capable of binding to laminin 5 and possibly to laminin 4, we wished to obtain direct evidence that ligation of $\alpha_6\beta_4$ at the cell surface is required for optimal phosphorylation of β_4 . A431 cells were plated for 60 min on the control ligand fibronectin, and then incubated for different times with polystyrene beads coated with the anti- β_4 mAb 3E1, the anti- β_1 mAb TS2/16, or the control anti-MHC mAb W6.32. As shown in Fig. 4, treatment with EGF caused significant tyrosine phosphorylation of β_4 in cells exposed for 10 min to the anti- β_4 beads, but not in cells treated with anti- β_1 or anti-MHC beads. Incubation with soluble 3E1 mAb produced a very modest effect. Control experiments indicated that treatment of A431 cells with anti- β_1 beads in the absence of EGF induces, as expected, a significant tyrosine phosphorylation of p125^{FAK} (data not shown). These results suggest that $\alpha_6\beta_4$ must be oligomerized at the cell surface to be susceptible to EGF-mediated phosphorylation.

Interestingly, while EGF could consistently induce significant tyrosine phosphorylation of β_4 in cells that had been plated onto a plastic culture substratum for 4 h or more (Fig. 3 *A*), maximal phosphorylation of β_4 occurred only transiently in cells plated on laminin 5 and 4 (Fig. 3 *B*) or incubated with anti- β_4 beads (Fig. 4). The transient nature of the effect induced by initial ligation of $\alpha_6\beta_4$ on tyrosine phosphorylation of β_4 may be explained by the pre-

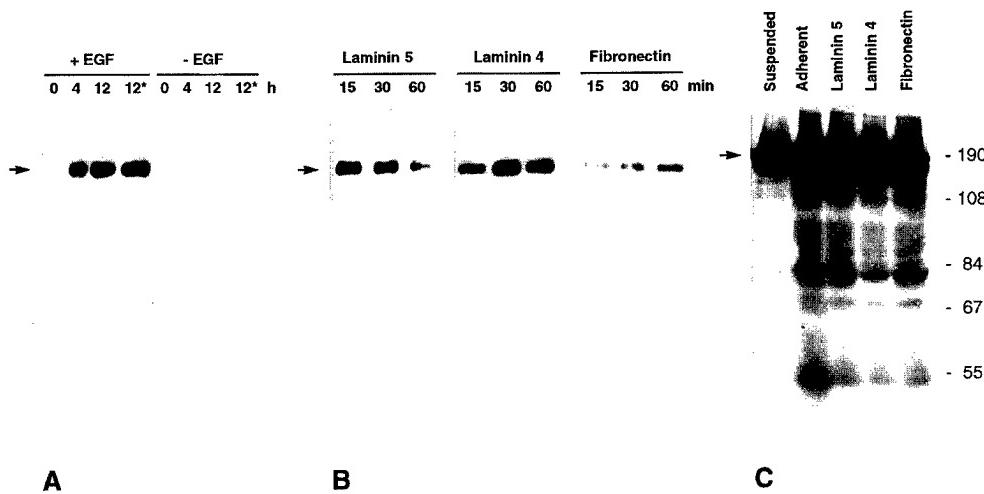


Figure 3. EGF-mediated tyrosine phosphorylation of β_4 requires extracellular matrix ligand binding. (*A*) A431 cells were detached, resuspended in complete medium, and either kept in suspension or replated at low density onto uncoated dishes for 4 or 12 h. Where indicated by the asterisk, the cells were plated at high density and reached confluence by 12 h. The cells were either left untreated or stimulated with 250 ng/ml EGF for 20 min, and then immunoprecipitated with the 3E1 mAb. Samples were probed by im-

munoblotting with polyclonal anti-P-Tyr antibodies. (*B*) A431 cells were serum starved, detached, and then replated in serum-free medium on dishes coated with laminin 5 matrix, 10 μ g/ml laminin 4, or 10 μ g/ml fibronectin for the indicated times. In all cases, the cells were treated with 50 ng/ml EGF for 5 min, and then immunoprecipitated with the 3E1 mAb. Samples were probed by immunoblotting with polyclonal anti-P-Tyr antibodies. (*C*) A431 cells were serum starved, detached, and then either kept in suspension or replated in serum-free medium on dishes coated with laminin 5 matrix, 10 μ g/ml laminin 4, or 10 μ g/ml fibronectin for 30 min. In all cases, the cells were treated with 50 ng/ml EGF for 5 min. Total proteins were probed by immunoblotting with polyclonal anti-P-Tyr antibodies.

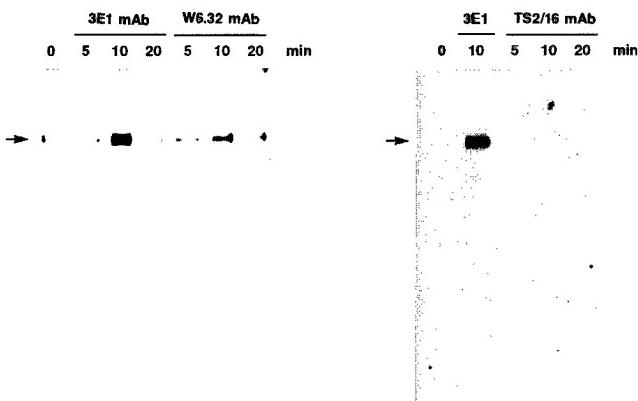


Figure 4. EGF-mediated tyrosine phosphorylation of β_4 requires ligation of the integrin. A431 cells were serum starved, detached, replated in serum-free medium on dishes coated with 10 $\mu\text{g}/\text{ml}$ fibronectin for 60 min, and then incubated for the indicated times with beads coated with the anti- β_4 mAb 3E1, the anti- β_1 mAb TS2/16, or the control anti-MHC mAb W6.32. In all cases, the cells were treated with 50 ng/ml EGF for 5 min, and then immunoprecipitated with the 3E1 mAb. The samples were probed by immunoblotting with polyclonal anti-P-Tyr antibodies.

vious observation that tyrosine phosphorylation of β_4 is negatively regulated by tyrosine phosphatases (Mainiero et al., 1995). Thus, although ligation of $\alpha_6\beta_4$ can activate a tyrosine kinase responsible for β_4 phosphorylation and synergize with the effect of EGF, the subsequent activation of tyrosine phosphatases able to reverse the phosphorylation of β_4 is likely to antagonize the effect of EGF. Taken together, these results suggest that clustering of $\alpha_6\beta_4$ induced by extracellular matrix ligands is required for optimal phosphorylation of the β_4 subunit in response to EGF stimulation. They further suggest that, depending on the timing, ligand binding to $\alpha_6\beta_4$ can either synergize or antagonize with a signal from the EGFR to induce tyrosine phosphorylation of β_4 .

EGF Induces Phosphorylation of Multiple β_4 Tyrosine Residues

Phosphopeptide mapping experiments were performed to analyze the β_4 sites phosphorylated in response to EGF treatment. Since cross-linking of $\alpha_6\beta_4$ by antibodies or plating on laminin 5 did not induce a level of tyrosine phosphorylation of β_4 sufficient for high resolution mapping, the sites phosphorylated in response to EGF were compared to those phosphorylated in response to pervanadate. Previous results have shown that treatment with pervanadate results in phosphorylation of multiple β_4 residues, including the β_4 TAM and presumably also the Shc binding sites, since pervanadate can induce association of $\alpha_6\beta_4$ with Shc (Mainiero et al., 1995). A431 cells were metabolically labeled with [^{32}P]orthophosphate, and then either left untreated or stimulated with EGF or pervanadate. After immunoprecipitation, the β_4 subunit was digested with *Staphylococcus* V8 protease, and the resulting peptides were separated by bidimensional TLC. As shown in Fig. 5 A, the β_4 subunit from unstimulated cells was resolved in a number of phosphopeptides (S1–S10). In accordance with the observation that β_4 is phosphorylated constitutively on

serine residues (Fig. 5 B), phosphoamino acid analysis indicated that these peptides contained only phosphoserine. Treatment with EGF resulted in the appearance of a number of additional phosphopeptides (Y1–Y8) (Fig. 5 B), and phosphoamino acid analysis of several of them (Y1–Y6) confirmed that they contained exclusively phosphotyrosine. These results indicate that exposure to EGF results in phosphorylation of multiple tyrosine residues in the β_4 cytoplasmic domain. The phosphopeptide map of β_4 from pervanadate-treated cells was similar, but not identical, to that of β_4 from EGF-stimulated cells. It contained the peptides S1–S10 and Y1–Y8, but also an additional phosphotyrosine-containing peptide, Y9. Furthermore, the intensity of the spot corresponding to phosphopeptide Y1 was much larger in pervanadate than in EGF-treated cells, and conversely, phosphopeptide Y6 was more intensely labeled in EGF than in pervanadate-treated cells (Fig. 5 C). Previous experiments of site-directed mutagenesis and phosphopeptide mapping of β_4 from pervanadate-treated cells have indicated that peptide Y5 contains tyrosine 1440, the COOH-terminal element of the TAM, and have provided circumstantial evidence that peptide Y2 contains tyrosine 1422, the NH₂-terminal element of the TAM (Mainiero et al., 1995). Since exposure to EGF resulted in the appearance of phosphopeptides Y5 and Y2, we concluded that EGF induces the phosphorylation of multiple tyrosine residues in β_4 and that these include the COOH-terminal, and possibly the NH₂-terminal, element of the TAM.

EGF-mediated Tyrosine Phosphorylation of β_4 Does Not Result in Recruitment of the Adaptor Proteins Shc and Grb2

To examine if EGF-mediated tyrosine phosphorylation of β_4 results in association of the adaptor protein Shc to $\alpha_6\beta_4$, A431 cells were either incubated with anti- β_4 beads in suspension or treated with EGF while adherent. The resulting extracts were immunoprecipitated with anti- β_4 antibodies and probed by immunoblotting with anti- β_4 and anti-Shc antibodies. As shown in Fig. 6 A, ligation of $\alpha_6\beta_4$ led to recruitment of Shc. In contrast, treatment with EGF did not result in association of this adaptor molecule to $\alpha_6\beta_4$. Immunoprecipitation with anti-Shc antibodies followed by immunoblotting with anti- β_4 antibodies confirmed that EGF stimulation does not result in recruitment of Shc to $\alpha_6\beta_4$ (Fig. 6 B). Control experiments indicated that a certain amount of the adaptor molecule remained available in the cytoplasm of EGF-treated cells (not shown; see also Fig. 6 C). The results of these experiments suggest that EGF does not induce phosphorylation of the Shc binding sites in β_4 .

The inability of EGF to induce association of Shc with the $\alpha_6\beta_4$ integrin raises the possibility that the EGFR and $\alpha_6\beta_4$ may, when simultaneously ligated, compete for this adaptor molecule in vivo. To explore this possibility, we examined the effect of EGF on the recruitment of Shc to activated $\alpha_6\beta_4$. As shown in Fig. 6 C, the amount of Shc coimmunoprecipitated with $\alpha_6\beta_4$ was lower in cells stimulated with anti- β_4 beads and EGF than in cells treated only with anti- β_4 beads. The inhibitory effect of EGF was especially evident in cells that had been incubated with anti- β_4 beads for 5 or 10 min, irrespective of whether the EGF was

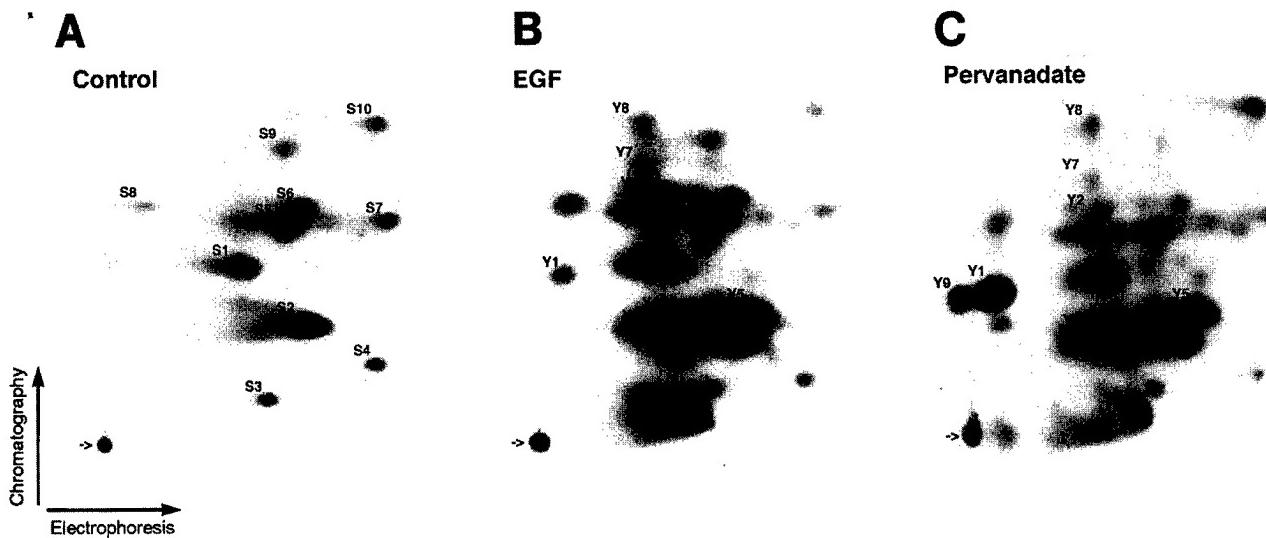


Figure 5. Phosphorylation of multiple β_4 tyrosine residues in response to EGF. A431 cells were metabolically labeled with [32 P]orthophosphate and left untreated (*A*), stimulated with 250 ng/ml EGF for 5 min (*B*), or with 500 μ M pervanadate for 10 min (*C*). After immunoprecipitation with the 3E1 mAb and separation by SDS-PAGE, the radioactive bands corresponding to β_4 were subjected to V8 protease digestion, and the resulting phosphopeptides were separated by bidimensional TLC.

applied together with the beads or before the beads. In addition to indicating that a certain amount of Shc remains available for binding to $\alpha_6\beta_4$ in EGF-treated cells, these results indicate that the EGFR and $\alpha_6\beta_4$ integrin compete

for this adaptor molecule in cultured cells, raising the possibility that the ability of $\alpha_6\beta_4$ to link to the *ras* pathway may be suppressed by EGF-dependent signals *in vivo*.

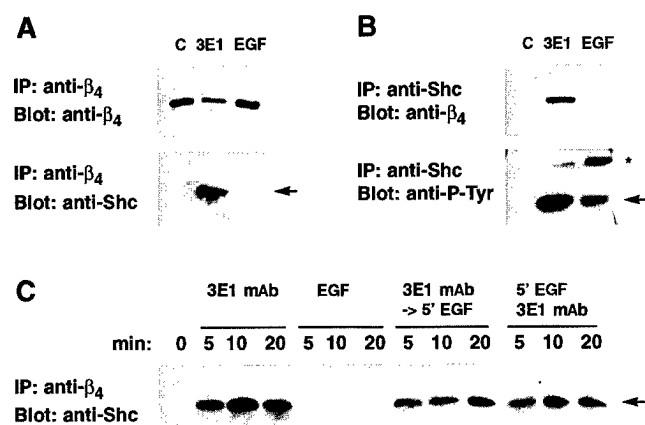


Figure 6. EGF interferes with the recruitment of Shc to $\alpha_6\beta_4$. (*A*) A431 cells were serum starved and either incubated for 10 min in suspension with polystyrene beads coated with the anti- β_4 mAb 3E1 or treated for 5 min while adherent with 200 ng/ml EGF. Control cells consisted of suspended cells left untreated. Equal amounts of total proteins were immunoprecipitated with anti- β_4 cytoplasmic peptide serum and probed by immunoblotting with the same antiserum (*top*) or anti-Shc mAb (*bottom*). (*B*) A431 cells were treated as above, but immunoprecipitated with anti-Shc polyclonal antibodies and probed with either anti- β_4 cytoplasmic peptide serum (*top*) or the anti-P-Tyr mAb PY20 (*bottom*). (*C*) A431 cells were serum starved, detached and replated in serum-free medium on dishes coated with 10 μ g/ml fibronectin for 60 min. They were then incubated for the indicated times with 3E1 mAb-coated beads, 50 ng/ml EGF, or 3E1 mAb-coated beads followed or preceded by a 5-min exposure to 50 ng/ml EGF. The extracts were immunoprecipitated with anti- β_4 cytoplasmic peptide antibody and probed by immunoblotting with anti-Shc mAb.

Disruption of Hemidesmosomes by EGF

To examine the effect of EGF on the ability of $\alpha_6\beta_4$ to associate with the hemidesmosomal cytoskeleton, we elected to use the rat 804G bladder epithelial cells, which form hemidesmosomes *in vitro*. Since these cells express very low levels of the EGFR, we used cell lines expressing moderate levels of human EGFR from cDNA. Immunoblotting analysis of total proteins with anti-P-Tyr antibodies indicated that exposure of the EGFR-transfected cells to EGF resulted in tyrosine phosphorylation of the recombinant EGFR and of several of its cellular substrates, including the β_4 subunit (data not shown and Fig. 2 *C, right*).

The EGFR-transfected 804G cells were starved, and then either left untreated or treated for various times with 100 ng/ml EGF. Immunofluorescent analysis revealed that while in control cells, $\alpha_6\beta_4$ and the BPAG 2 were concentrated at the basal cell surface within Triton X-100-resistant, "Swiss cheese"-like structures corresponding to hemidesmosomes (Fig. 7, *a* and *b*); in cells treated with EGF, these molecules had undergone a profound redistribution and were no longer detected in association with these structures (Fig. 7, *d* and *e*). To confirm the physiological significance of these observations, we examined the effect of EGF on the hemidesmosome-like structures formed by normal human primary keratinocytes in culture. As shown in Fig. 7 (*c* and *f*), treatment with EGF resulted in loss of hemidesmosomal staining also in these cells, suggesting that disassembly of hemidesmosomes may be one of the physiological consequences of activation of the EGFR in primary epithelial cells. Immunofluorescent analysis of EGFR transfected 804G cells treated for various times with EGF indicated that the effect of the growth factor on hemidesmosomes was already significant after 1 h and

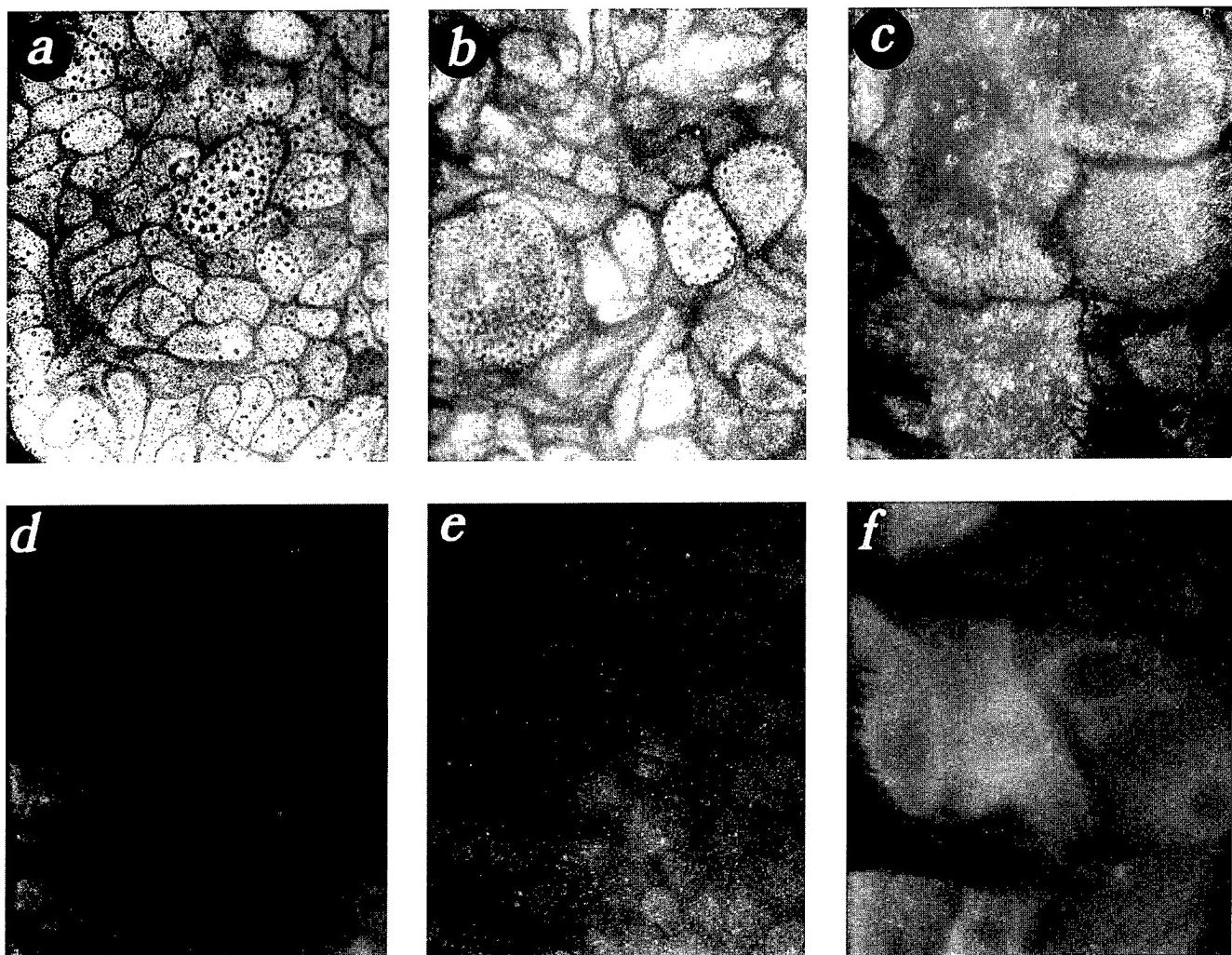


Figure 7. Disruption of hemidesmosomes by EGF. EGFR-transfected 804G cells (*a*, *b*, *d*, and *e*) and primary human keratinocytes (*c* and *f*) were cultured on glass coverslips for 48 h, serum starved, and either left untreated (*a*–*c*) or treated with 100 ng/ml EGF for 12 h (*d*–*f*). After extraction with 0.2% Triton X-100, the cells were fixed and stained with anti-BPAG 2 antibodies (*a* and *d*), anti- β_4 cytoplasmic peptide serum (*b* and *e*), or the anti- β_4 mAb 3E1 (*c* and *f*) followed by FITC-labeled affinity-purified secondary antibodies.

complete by 12 h of treatment (Fig. 8, *a*–*e*). Dose-dependency experiments indicated that 25 ng/ml of EGF were sufficient to induce a significant effect on hemidesmosomes (data not shown). The effect of EGF was specific as PDGF did not cause any change in hemidesmosome staining (Fig. 8 *f*). Similar results were obtained with three independent clones of EGFR-transfected 804G cells. These observations indicate that EGF treatment causes disruption of hemidesmosomes.

The ability of EGF to induce hemidesmosome disassembly was unexpected because phosphopeptide mapping had indicated that the β_4 TAM, which mediates a signaling event required for the association of $\alpha_6\beta_4$ with hemidesmosomes (Mainiero et al., 1995), is phosphorylated in response to EGF. We wondered if the effect of EGF on hemidesmosomes was caused by its ability to downregulate ligand binding to $\alpha_6\beta_4$ by a mechanism of inside-to-outside signaling. The effect of EGF treatment on the adhesion of EGFR-transfected 804G cells to laminin 4 and 5 was therefore examined. To block β_1 -dependent adhesion, the cells were plated on the two extracellular matrix

proteins in the presence of inhibitory anti- β_1 antibodies. As shown in Fig. 9, the extent to which the EGFR-transfected 804G cells adhered to laminin 4 and 5 was not significantly changed after treatment with EGF. A similar result was obtained with A431 cells (not shown). These results indicate that exposure to EGF does not cause a significant change in ligand binding to $\alpha_6\beta_4$, thus suggesting that the deterioration of hemidesmosomes observed in EGF-treated cells is not caused by a downregulation of ligand binding. Together with the observation that EGF induces phosphorylation of the β_4 TAM, these data suggest the hypothesis that EGF-dependent signals suppress the association of $\alpha_6\beta_4$ with the hemidesmosomal cytoskeleton by interfering with the functioning of signaling and cytoskeletal molecules downstream of the β_4 TAM.

Increased $\alpha_6\beta_4$ -dependent Cell Migration in Response to EGF

To determine if the apparent disruption of hemidesmosomes caused by EGF correlates with a change in $\alpha_6\beta_4$ -

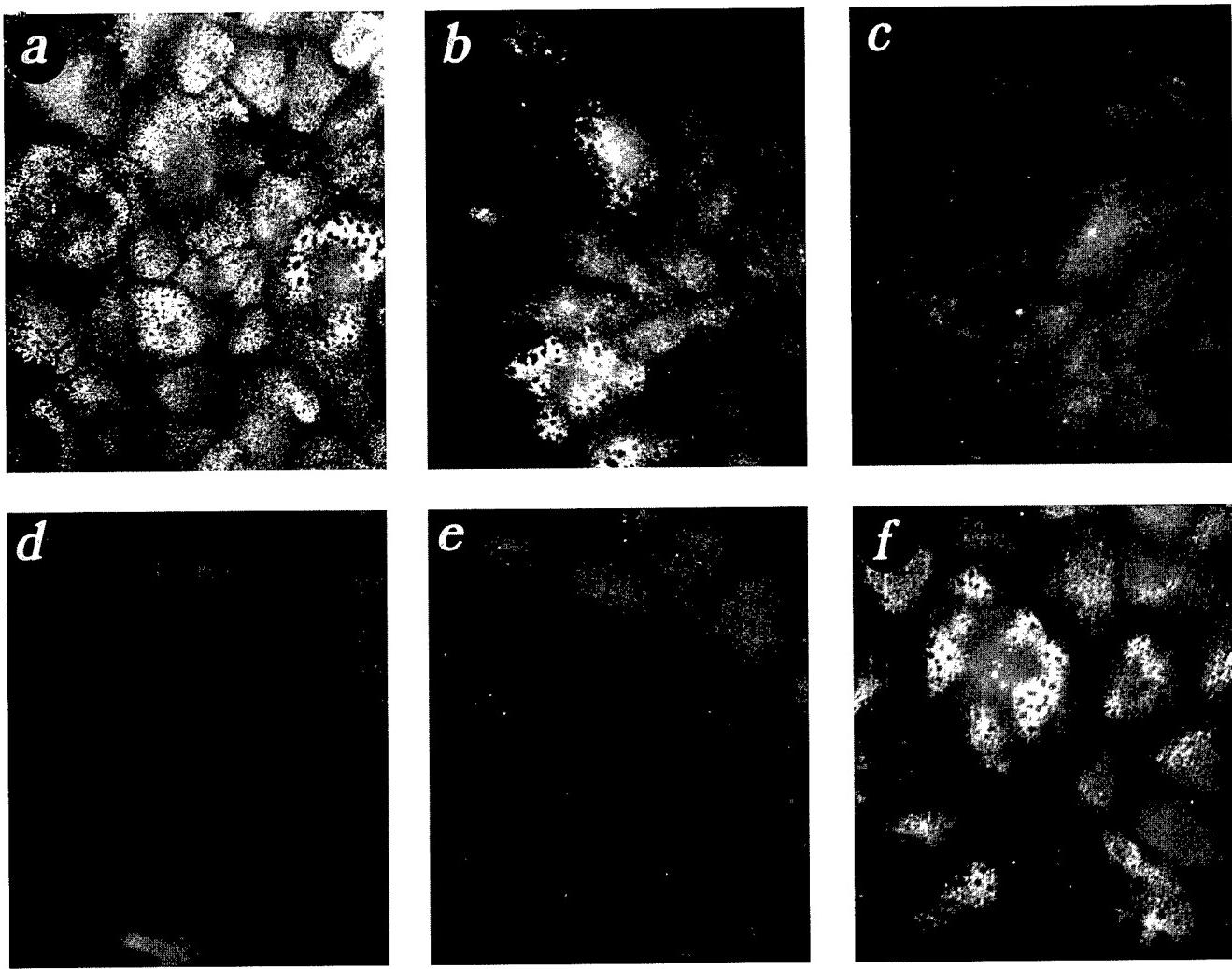


Figure 8. Kinetics and specificity of EGF-induced hemidesmosome disruption. EGFR-transfected 804G cells were cultured on glass coverslips for 48 h, serum starved, and either left untreated (*a*) or treated with 100 ng/ml EGF for 30 min (*b*), 1 h (*c*), 3 h (*d*), or 12 h (*e*). As a control, cells were exposed to 5 ng/ml PDGF for 12 h (*f*). After extraction with 0.2% Triton X-100, the cells were fixed and stained with anti-BPAG 2 antibodies followed by FITC-labeled affinity-purified secondary antibodies.

dependent cell migration, we measured the ability of control and EGFR-transfected 804G cells to migrate toward various extracellular matrix components by using a Boyden chamber system. As shown in Fig. 10, treatment with EGF resulted in increased migration of the EGFR-transfected 804G cells toward the two $\alpha_6\beta_4$ ligands laminin 4 and 5, but not the control ligand fibronectin, suggesting that EGF-dependent signals can increase cell migration toward $\alpha_6\beta_4$ ligands. In addition, the basal migration of EGFR-transfected 804G cells toward laminin 4 and 5 was greater than that of control 804G cells. This result suggests that the recombinant EGFR may be partially active in the absence of exogenous ligand in 804G cells, perhaps because these cells secrete EGF or TGF- α . In accordance with this hypothesis, we found that the medium conditioned by 804G cells is capable of stimulating the auto-phosphorylation of recombinant EGFR expressed in transfected 804G cells (data not shown). Inhibitory anti- β_1 antibodies were able to suppress the migration of unstimulated cells toward laminin 5 by $91 \pm 6\%$, but only inhibited the migration of EGF-treated cells by $11 \pm 3\%$, indicating

that the EGF-stimulated migration toward laminin 5 was largely dependent on $\alpha_6\beta_4$ function. This conclusion was also supported by the observation that EGFR-transfected NIH-3T3 fibroblasts, which do not express $\alpha_6\beta_4$, did not respond to EGF with increased migration toward laminin 4 (Fig. 10, top). Finally, the effect of EGF was specific, since it was not observed in response to PDGF or with the control 804G cells in response to EGF. Taken together, these results indicate that EGF specifically upregulates $\alpha_6\beta_4$ -dependent migration toward laminins.

Discussion

Several observations suggest that $\alpha_6\beta_4$ - and growth factor-dependent signals may cooperate to control epidermal cell proliferation and migration. In stratified epithelia, such as the epidermis, $\alpha_6\beta_4$ mediates the interaction of basal keratinocytes with the basement membrane (Kajiji et al., 1989), and there is evidence indicating that these cells have to remain in contact with this extracellular matrix to maintain their proliferative potential (Green, 1977; Hall and

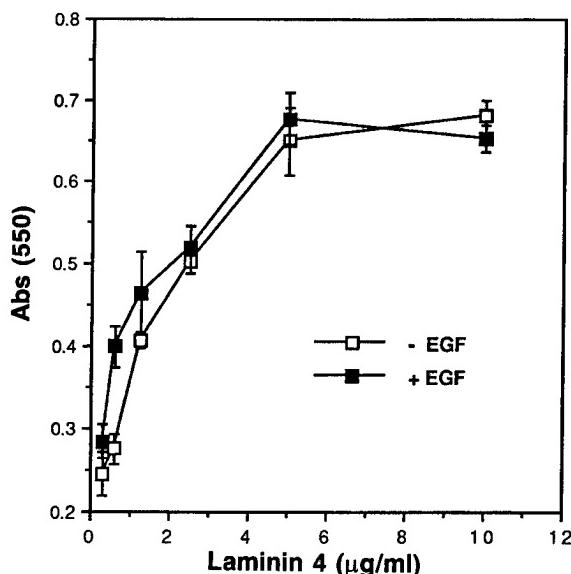


Figure 9. EGF does not affect $\alpha_6\beta_4$ -dependent cell adhesion. EGFR-transfected 804G cells were starved and either left untreated or stimulated with 100 ng/ml EGF. The cells were plated in the presence of inhibitory anti- β_1 antibodies on dishes coated with the indicated amounts of laminin 4 for 60 min (top) or on laminin 5 matrix-coated dishes for the indicated times (bottom).

Watt, 1989). Furthermore, the coincident expression of $\alpha_6\beta_4$ and laminins by keratinocytes migrating into corneal wounds suggests a role for $\alpha_6\beta_4$ -mediated migration during the reepithelialization of wounds (Kurpakus et al., 1991). Prompted by the prominent role of EGF and transforming growth factor α in controlling keratinocyte growth and migration (Rheinwald and Green, 1977; Barrandon and Green, 1987), and by the coincident expression of $\alpha_6\beta_4$ and EGFR in basal keratinocytes *in vivo* (Green et al., 1987; Kajiji et al., 1989), we have examined the effect of EGFR activation on the intracellular functions of $\alpha_6\beta_4$. Our results indicate that EGF-dependent signals have a complex effect on $\alpha_6\beta_4$ function: they cause tyrosine phosphorylation of β_4 without promoting the association of Shc, induce

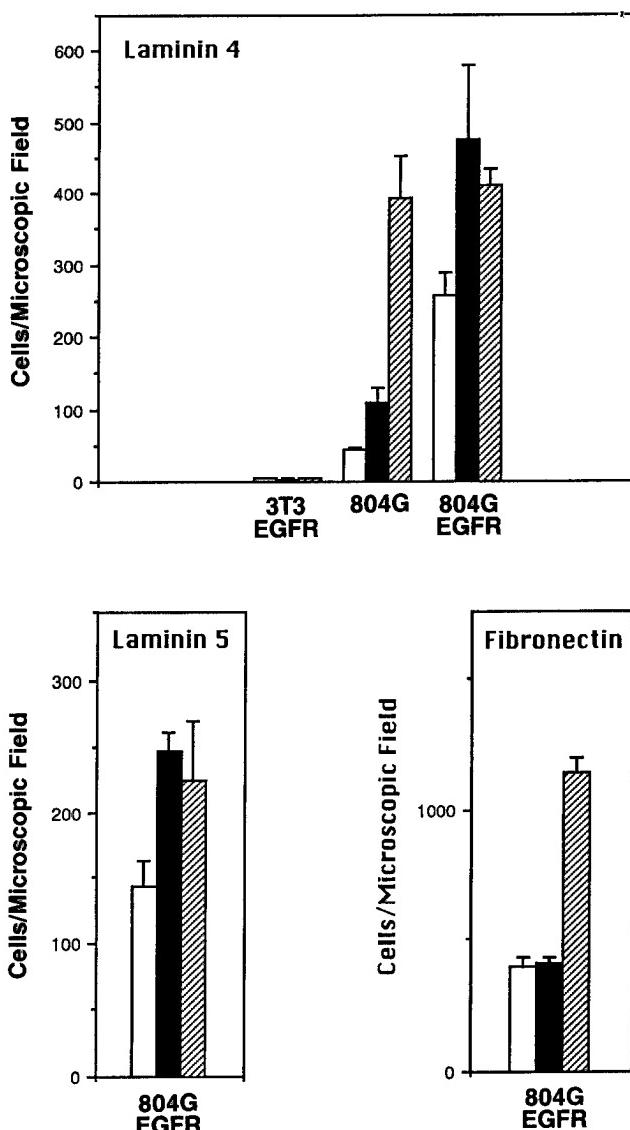


Figure 10. EGF stimulates $\alpha_6\beta_4$ -dependent cell migration. The indicated cell lines were allowed to migrate toward laminin 4 for 48 h, laminin 5 for 12 h, and fibronectin for 48 h in the presence of control medium (open bars), 50 ng/ml EGF (closed bars), or 5 ng/ml PDGF (hatched bars).

disassembly of hemidesmosomes, and upregulate cell migration on laminins.

In this study, we provide direct evidence that activation of the EGFR causes tyrosine phosphorylation of the β_4 subunit. This phosphorylation is characterized by a rapid kinetics and, at least in A431 cells, by a high stoichiometry. Since we have been unable to obtain evidence that the EGFR efficiently phosphorylates β_4 *in vitro*, it is our hypothesis that the EGFR does not directly phosphorylate β_4 *in vivo*, but rather it activates a signaling pathway that results in its phosphorylation. The observation that EGF-mediated phosphorylation of β_4 requires ligation of the integrin by extracellular ligand or antibodies suggests that this phosphorylation event is mediated by an integrin-associated kinase acting in trans. Future studies will be required to determine if $\alpha_6\beta_4$ is indeed an indirect target of

the EGFR and if it is associated with two distinct tyrosine kinases, one activated by EGF and the other by extracellular matrix binding, or with a single tyrosine kinase activated by both stimuli.

The results of phosphopeptide mapping indicate that EGF causes phosphorylation of several distinct β_4 tyrosine residues. Although the majority of the tyrosine phosphorylation sites in β_4 remain to be identified and their function assessed, the complexity of the tyrosine phosphorylation pattern induced by EGF suggests that many $\alpha_6\beta_4$ functions may be regulated by the growth factor. One major intracellular function of $\alpha_6\beta_4$ that is negatively regulated by EGF is the recruitment of the adaptor molecule Shc. Treatment with EGF does not result in the association of $\alpha_6\beta_4$ with Shc and presumably Grb2. In fact, exposure to EGF partially suppresses the recruitment of Shc to the ligated integrin. Although it is possible that EGF causes a conformational change or another posttranslational modification of $\alpha_6\beta_4$ that prevents it from binding to Shc, the most likely explanation of these results is that the growth factor does not induce phosphorylation of the Shc binding motifs in β_4 . The observation that the EGFR can compete with $\alpha_6\beta_4$ for the recruitment of Shc is in accordance with the recognized ability of activated EGFR to associate with this adaptor molecule (Pellicci et al., 1992) and suggests that a significant activation of the EGFR may interfere with the ability of ligand-occupied $\alpha_6\beta_4$ to activate signaling *in vivo*. In contrast, when suboptimally ligated, the EGFR and $\alpha_6\beta_4$ are likely to cooperate with each other to activate the *ras* pathway. This latter prediction may be relevant to understanding anchorage-dependent cell growth in epithelial cells.

The results of our immunofluorescent analysis indicate that treatment with EGF causes disruption of hemidesmosomes in both EGFR-transfected 804G cells and primary human keratinocytes. What is the mechanism by which EGF interferes with the assembly of hemidesmosomes? Our previous studies suggest that the nucleation of hemidesmosomes requires a signal mediated by the β_4 TAM (Mainiero et al., 1995). It is, however, unlikely that the phosphorylation of the TAM is the only $\alpha_6\beta_4$ function necessary for the assembly of hemidesmosomes. Deletion mutagenesis experiments have indicated that the association of $\alpha_6\beta_4$ with the hemidesmosomal cytoskeleton not only requires the connecting segment, which includes the TAM, but also sequences within the two type III fibronectin-like modules upstream of the connecting segment (Spinardi, L., and F.G. Giancotti, unpublished results). This observation is consistent with the hypothesis that a TAM-dependent signal renders one or more cytoskeletal elements of hemidesmosomes competent for binding to sequences within the first two type III fibronectin-like modules of β_4 . Further assembly of hemidesmosomes may then be driven by the cooperative binding of additional cytoskeletal elements. Based on this model, EGF-dependent signals may interfere with the assembly of hemidesmosomes at one or more of several steps. Since EGF does not affect $\alpha_6\beta_4$ -mediated adhesion to laminins and does not suppress phosphorylation of the β_4 TAM, the growth factor may interfere with the functioning of one or more signaling or cytoskeletal molecules located downstream of the TAM in the pathway that controls the association of

$\alpha_6\beta_4$ with the cytoskeleton. Furthermore, it is possible that EGF induces the phosphorylation of tyrosine residues located within the first two type III fibronectin-like modules of the β_4 tail, thus directly interfering with the association of cytoskeletal molecules. Finally, as the process of hemidesmosome formation is likely to be complex and to require the function of many components in addition to $\alpha_6\beta_4$ and the molecules to which it binds, EGF may disrupt hemidesmosomes by acting on one or more of these additional components.

Most of the previous studies on the regulation of the cytoskeleton by growth factors have focused on the effects of EGF and PDGF on the actin filament system. It has been known for long that these growth factors can induce profound changes in the architecture of the actin cytoskeleton (Bockus and Stiles, 1984; Herman and Pledger, 1985). Recent studies have indicated that they can induce the sequential formation of filopodia, lamellipodia, and focal adhesions, and that these cytoskeletal changes are mediated by a GTPase cascade involving Cdc 42, Rac, and Rho (Nobes and Hall, 1995). Our current observations clearly indicate that EGF can also profoundly affect the keratin filament system, thereby providing evidence for a novel mechanism of cytoskeletal regulation by EGF.

The changes in the association of $\alpha_6\beta_4$ with the cytoskeleton induced by activated EGFR are likely to be significant in both physiological and pathological situations. Several lines of evidence support the notion that hemidesmosomes mediate stable adhesion to the basement membrane (Uitto and Christiano, 1992; Guo et al., 1995; Spinardi et al., 1995). Their disruption may therefore result in a more dynamic interaction with the extracellular matrix. In accordance with this hypothesis, we have observed that the disassembly of hemidesmosomes caused by EGF correlates with an increase in $\alpha_6\beta_4$ -dependent cell migration. This observation suggests that the ability of $\alpha_6\beta_4$ to mediate cell migration on laminins can be upregulated by factors that interfere with its association with the hemidesmosomal cytoskeleton. It is well known that EGF and TGF- α can promote the reepithelialization of wounds (Schultz et al., 1991), and it has recently been observed that keratinocytes lose their hemidesmosomes as they migrate into corneal wounds (Gipson et al., 1993). Thus, the ability of activated EGFR to coordinately disassemble hemidesmosomes and increase cell migration on laminins is likely to be important during wound healing. In addition, there is evidence indicating that keratinocytes of patients affected by the skin disease psoriasis overproduce TGF- α (Elder et al., 1989) and that squamous carcinoma cells overexpress the EGFR (Yamamoto et al., 1986; Ozanne et al., 1986). In both pathological situations, the expression of $\alpha_6\beta_4$ is no longer restricted to the basal surface of those cells that abut the basement membrane, but extends suprabasally (Kimmel and Carey, 1986; Pellegrini et al., 1992). Our current results suggest that the loss of $\alpha_6\beta_4$ polarity observed in these diseases may result from the ability of activated EGFR to disrupt the association of the integrin to the hemidesmosomal cytoskeleton. They further suggest that the ability of EGFR to affect the association of $\alpha_6\beta_4$ with the cytoskeleton may contribute to the invasive ability of squamous carcinoma cells.

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The Adaptor Protein Shc Couples a Class of Integrins to the Control of Cell Cycle Progression

Kishore K. Wary,^{*†} Fabrizio Mainiero,^{*‡}
Steven J. Isakoff,[§] Eugene E. Marcantonio,^{||}
and Filippo G. Giancotti^{*†}

^{*}Department of Pathology and Kaplan Cancer Center

[§]Sackler Institute of Graduate Biomedical Sciences
New York University School of Medicine

New York, New York 10016

^{||}Department of Pathology

College of Physicians and Surgeons

Columbia University

New York, New York 10032

Summary

We provide evidence that a class of integrins combines with the adaptor Shc and thereby with Grb2. Coimmunoprecipitation and mutagenesis experiments indicate that the recruitment of Shc is specified by the extracellular or transmembrane domain of integrin α subunit and suggest that this process is mediated by caveolin. Mutagenesis and dominant-negative inhibition studies reveal that Shc is necessary and sufficient for activation of the MAP kinase pathway in response to integrin ligation. Mitogens and Shc-activating integrins cooperate to promote transcription from the Fos serum response element and transit through G1. In contrast, adhesion mediated by integrins not linked to Shc results in cell cycle arrest and apoptosis even in presence of mitogens. These findings indicate that the association of specific integrins with Shc regulates cell survival and cell cycle progression.

Introduction

In addition to promoting cell adhesion, the extracellular matrix exerts complex and often divergent effects on cellular behavior. Normal cells require anchorage to the extracellular matrix in order to proliferate, and loss of this growth control mechanism is a hallmark of neoplastic cells (Giancotti and Mainiero, 1994). In a number of settings, however, interaction with the extracellular matrix promotes exit from the cell cycle and differentiation (Lin and Bissell, 1993). Recent results suggest that adhesion to the extracellular matrix may also be required for cell survival (Ruoslahti and Reed, 1994). These diverse activities of the extracellular matrix are likely to be mediated by the ability of integrins to activate intracellular signaling pathways, but the mechanisms involved are incompletely understood.

In vertebrates, cell adhesion to the extracellular matrix is mediated by at least 15 distinct integrins, including

nine β_1 and four α_v subunit-containing heterodimers (Ruoslahti, 1991; Hynes, 1992). Individual integrins can recognize several extracellular matrix molecules; conversely, one extracellular matrix molecule usually binds to several integrins. Despite this high degree of apparent redundancy, several integrin gene knockouts cause developmental abnormalities. In particular, mutation of each of the three major fibronectin-binding integrins produces a distinct phenotype, suggesting that integrins with overlapping ligand binding specificities carry out distinct functions (Hynes, 1996). Although differences in ligand binding affinity and association with the cytoskeleton have been demonstrated, the observation that the extracellular matrix can promote either proliferation or growth arrest and differentiation, depending on its composition and the cell type involved (Lin and Bissell, 1993), suggests the existence of signaling differences among integrins.

The intracellular signals elicited by β_1 and α_v integrins resemble those induced by receptor tyrosine kinases, suggesting that tyrosine phosphorylation plays a crucial role in integrin signaling (Clark and Brugge, 1995). Recent studies on integrin signaling have focused on the tyrosine kinase named focal adhesion kinase (FAK) (Schaller and Parsons, 1994). FAK can interact with signaling molecules capable of regulating gene expression (Chen and Guan, 1994; Schlaepfer et al., 1994; Vuori et al., 1996). However, the effects of disrupting FAK by gene targeting or dominant-negative inhibition appear to be limited to the cytoskeleton (Ilić et al., 1995; Richardson and Parsons, 1996). Furthermore, because all β_1 and α_v integrins are able to stimulate FAK, the activation of FAK does not explain the differential effects of extracellular matrix on cellular function.

In this paper, we demonstrate that a subset of β_1 and α_v integrins are linked to the MAP kinase pathway by the adaptor protein Shc, and we provide evidence that this signaling mechanism regulates cell survival and cell cycle progression in response to the extracellular matrix.

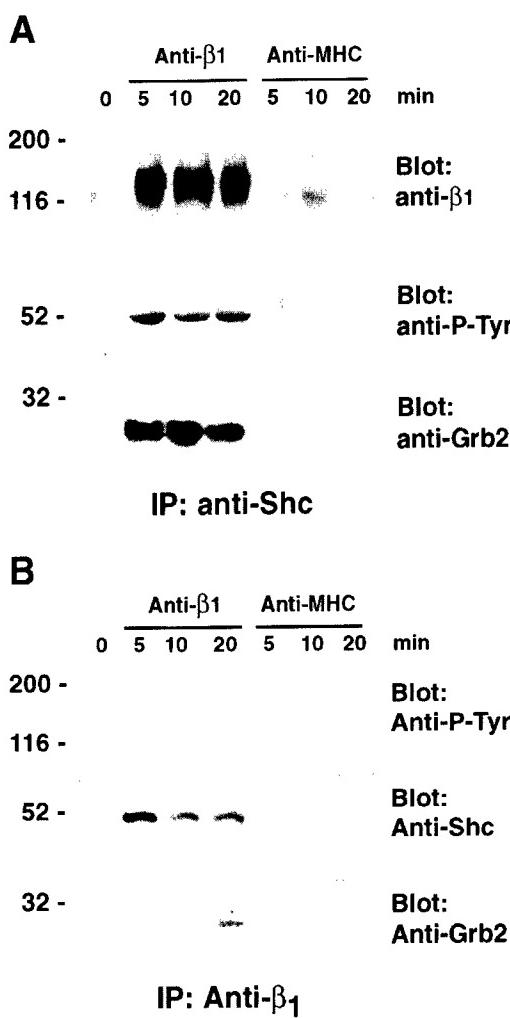
Results

Association of β_1 Integrins with Shc and Grb2

Preliminary experiments indicated that cell adhesion to fibronectin results in tyrosine phosphorylation of proteins with apparent molecular masses of 125 kDa, 80 kDa, 52 kDa, and 46 kDa. While the 125 kDa and 80 kDa proteins comigrated with FAK and paxillin, the 52 kDa and 46 kDa proteins displayed a mobility similar to that of the two major isoforms of Shc. Shc is a SH2-phosphotyrosine-binding (PTB) domain adaptor that links tyrosine kinases to Ras signaling by recruiting the Grb2-mSOS complex to the plasma membrane in a tyrosine phosphorylation-dependent manner (Pawson, 1995). To test whether antibody-mediated ligation of β_1 integrins resulted in tyrosine phosphorylation of Shc and association of Shc with Grb2, A431 cells were incubated in suspension with polystyrene beads coated with

[†]Present address: Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10021.

[‡]Present address: Dipartimento di Patologia Sperimentale e Medicina, Università La Sapienza, Viale Regina Elena 324, 00161 Rome, Italy.

Figure 1. Recruitment of Shc and Grb2 by β_1 Integrins

(A) A431 cells were stimulated in suspension with 4B4 (anti- β_1) or W6.32 (anti-MHC) MAb-coated beads for the indicated times. Equal amounts of proteins were immunoprecipitated with an antiserum reacting predominantly with p52^{shc} and subjected to immunoblotting with anti- β_1 cytoplasmic domain serum, anti-P-Tyr MAb PY20, or anti-Grb2 MAb EL6.

(B) A431 cells were stimulated as above, immunoprecipitated with anti- β_1 MAb TS2/16, and subjected to immunoblotting with anti-P-Tyr MAb PY20, anti-Shc serum, or anti-Grb2 serum.

the anti- β_1 monoclonal antibody (MAb) 4B4 or the control anti-major histocompatibility complex (MHC) MAb W6.32 and immunoprecipitated with an antiserum reacting predominantly with the 52 kDa isoform of Shc. Immunoblotting with anti-phosphotyrosine (anti-P-Tyr) and anti-Grb2 antibodies indicated that cross-linking of β_1 integrins, but not MHC molecules, results in tyrosine phosphorylation of p52^{shc} and association of Shc with Grb2 (Figure 1A).

To examine whether β_1 integrins formed a complex with Shc, anti-Shc immunoprecipitates were subjected to immunoblotting with anti- β_1 antibodies, and anti- β_1 immunoprecipitates were probed by immunoblotting with anti-Shc antibodies. The results indicated that β_1 integrins associate with Shc in response to stimulation

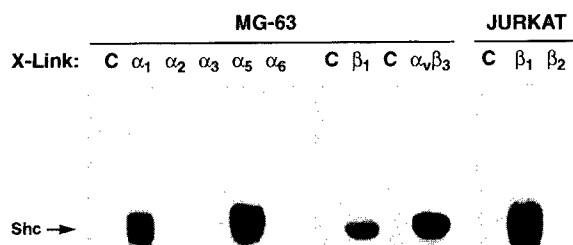


Figure 2. Spectrum of Integrins Linked to Shc

Cells were stimulated in suspension with W6.32 (c), 4B4 (β_1), P4H9 (β_2), LM609 ($\alpha_v\beta_3$), TS2/7 (α_1), PIE6 (α_2), PIB5 (α_3), PID6 (α_5), or GoH3 (α_6) MAb-coated beads, immunoprecipitated with anti-Shc serum, and subjected to immunoblotting with anti-P-Tyr MAb PY20.

with anti- β_1 but not anti-MHC beads (Figure 1). In accordance with the hypothesis that the association of Grb2 with β_1 integrins is predominantly mediated by Shc, we detected a lower amount of Grb2 in anti- β_1 than anti-Shc immunoprecipitates (compare Figure 1A with Figure 1B).

Since the β_1 tail contains two tyrosines (Y783 and Y795) that upon phosphorylation could provide a binding site for the PTB domain of Shc (N-P-X-pY), we tested whether the interaction of β_1 integrins with Shc was mediated by tyrosine phosphorylation of the β_1 tail. However, experiments of immunoblotting with anti-P-Tyr antibodies failed to demonstrate tyrosine phosphorylation of β_1 integrins in cells stimulated with anti- β_1 beads (Figure 1B). In addition, Sepharose beads carrying GST fusion proteins encoding the PTB or SH2 domains of Shc did not bind the β_1 subunit from SDS-denatured extracts of anti- β_1 -stimulated cells (data not shown). Taken together, these results are consistent with the hypothesis that β_1 integrins interact with Shc indirectly.

Since Shc potentially links β_1 integrins to Ras signaling, we examined whether ligation of β_1 integrins activated Ras. In vivo Ras-GTP loading experiments indicated that adhesion of primary human keratinocytes to anti-MHC MAb-coated dishes does not result in a significant activation of Ras (18.5% GTP/GTP plus GDP). In contrast, adhesion to anti- β_1 MAb-coated dishes caused activation of Ras to a level (43% GTP/GTP plus GDP) comparable to that induced by treatment with tetradecanoyl phorbol acetate (TPA) and ionomycin (49% GTP/GTP plus GDP). These findings suggest that the recruitment of Shc by β_1 integrins contributes to the activation of Ras in response to cell adhesion.

The Association with Shc Defines a Class of Integrins

To determine the spectrum of integrins linked to Shc, we examined Jurkat cells, which express various β_1 and β_2 integrins, and MG-63 cells, which express the promiscuous integrins $\alpha_v\beta_3$ and $\alpha_3\beta_1$, the collagen/laminin receptors $\alpha_1\beta_1$ and $\alpha_2\beta_1$, the fibronectin receptor $\alpha_5\beta_1$, and the laminin receptor $\alpha_6\beta_1$. As shown in Figure 2, antibody-mediated cross-linking of $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ caused tyrosine phosphorylation of Shc. In contrast,

ligation of $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, and β_2 integrins did not induce this event. Since the beads coated with anti- α_2 , - α_3 , and - α_6 MAbs were able to induce tyrosine phosphorylation of FAK (Figure 4C; data not shown), it is unlikely that they mediate inefficient cross-linking or interfere with some other aspect of integrin signaling. Furthermore, beads carrying three different MAbs to the ectodomain of α_6 , including two that interfere with the adhesive function of $\alpha_6\beta_1$ and one that does not, failed to induce tyrosine phosphorylation of Shc, while beads coated with three MAbs to α_5 , including both adhesion-blocking and -nonblocking reagents, consistently induced this event (data not shown). These results suggest that $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$ are unable to combine with Shc and induce its tyrosine phosphorylation. In accordance with this conclusion, coimmunoprecipitation analysis indicated that $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$, but not $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$, associate with Shc upon antibody-mediated cross-linking (see Figures 4A and 4C; data not shown). These findings indicate that integrins with overlapping binding specificity can be distinguished on the basis of their ability to combine with Shc and induce its phosphorylation.

The Association with Shc Is Specified by the Extracellular or Transmembrane Domain of Integrin α Subunit

To define the integrin sequences involved in the recruitment of Shc, we examined NIH 3T3 and Chinese hamster ovary (CHO) cells expressing the recombinant human wild-type and mutant integrin subunits illustrated in Figure 3. Fluorescence-activated cell sorter (FACS) analysis indicated that each mutant integrin subunit was expressed at a level comparable to that of the corresponding recombinant wild-type control. The cells were stimulated with anti-integrin or control MAb-coated beads and subjected to immunoprecipitation and immunoblotting analysis. The results indicated that neither a simultaneous phenylalanine substitution in correspondence of both N-P-X-Y motifs ($\beta_1Y783F-Y795F$) nor a deletion of almost the entire cytoplasmic portion of β_1 ($\beta_1\Delta$ Cyto) affects the ability of β_1 integrins to associate with Shc (Figure 4A) and induce its phosphorylation (data not shown), confirming the hypothesis that the β_1 cytoplasmic domain does not interact with Shc in a tyrosine phosphorylation-dependent manner and indicating that the majority of the β_1 tail is not required for association with Shc. To our surprise, neither a deletion of the α_1 cytoplasmic domain immediately following the conserved GFFKR box ($\alpha_1\Delta$ Cyto) nor a deletion of the entire α_5 tail ($\alpha_5\Delta$ Cyto) diminished the ability of $\alpha_1\beta_1$ and $\alpha_5\beta_1$, respectively, to associate with Shc (Figure 4A) and promote its phosphorylation (data not shown). These results indicate that the cytoplasmic domain of α subunit, like the corresponding portion of β subunit, is not required for recruitment and tyrosine phosphorylation of Shc.

To exclude the possibility that each cytoplasmic tail was independently capable of recruiting Shc, 293-T cells were transiently transfected with vectors encoding single-subunit chimeras consisting of the extracellular and transmembrane portion of the interleukin-2 receptor α chain linked to the cytoplasmic tail of either the α_5 (Tac- α_5) or β_1 integrin subunit (Tac- β_1). As shown in Figure

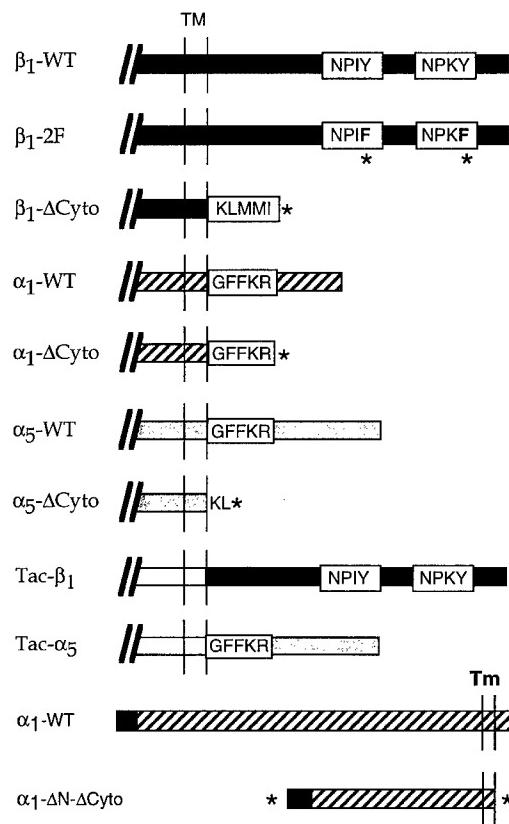


Figure 3. Schematic Representation of Recombinant Wild-Type and Mutant Human Integrin Subunits

Asterisks point to amino acid substitutions and deletions. α_1 -WT and α_1 - Δ N- Δ Cyto are in smaller scale than other mutants.

4B, neither cross-linking of Tac- α_5 nor of Tac- β_1 caused recruitment of Shc. Even coclustering of the two chimeras was not sufficient to induce recruitment of Shc. In contrast, ligation of endogenous wild-type β_1 integrins in mock-transfected cells resulted, as expected, in efficient recruitment of Shc. Taken together, the results of this mutational analysis suggest that the recruitment of Shc and its tyrosine phosphorylation are not mediated by the cytoplasmic domains of the integrin α or β subunits.

To explore further the mechanism of recruitment and phosphorylation of Shc in response to integrin stimulation, we examined the properties of a mutant human α_1 subunit that carries a deletion of the N-terminal domain involved in association with β_1 and of the C-terminal tail (see Figure 3). Despite its inability to combine with β_1 , this mutant subunit is efficiently exported to the cell surface (Kern et al., 1994). As shown in Figure 4C, ligation of single-chain tailless α_1 caused recruitment and tyrosine phosphorylation of Shc as effectively as stimulation of wild-type $\alpha_1\beta_1$, while ligation of endogenous $\alpha_6\beta_1$ did not induce these events in both transfectants. These results indicate that the association of $\alpha_1\beta_1$, and presumably also $\alpha_5\beta_1$ and $\alpha_v\beta_3$, with Shc is specified by sequences contained in the membrane-proximal portion of the extracellular domain of the α subunit, its transmembrane segment, or both.

Association of Integrins with Caveolin

Because Shc is a cytosolic protein, the results described above suggest that the association of integrins with Shc is mediated by an intermediary membrane component. Caveolin is a 22 kDa transmembrane protein that links a variety of cell-surface receptors lacking a cytoplasmic domain to intracellular signaling pathways (Lisanti et al., 1994). We wished therefore to examine whether caveolin associated with β_1 integrins and Shc. The A431 cells were incubated with anti-MHC or anti- β_1 beads and immunoprecipitated with antibodies to caveolin, Shc, and β_1 integrins. The samples were probed by immunoblotting with antibodies reacting with the β_1 subunit or the three isoforms of Shc. The results revealed that caveolin is constitutively associated with β_1 integrins and forms a complex with Shc upon stimulation with anti- β_1 beads (Figure 5A). The anti-caveolin immunoprecipitates contained similar amounts of the precursor and mature form of β_1 , suggesting that β_1 integrins associate with caveolin in the endoplasmic reticulum. To obtain evidence that the association of β_1 integrins with caveolin was not an artifact of cell lysis, we performed immunofluorescent staining of primary human fibroblasts. The results indicated that a significant fraction of caveolin is not diffusely distributed at the cell surface, but colocalizes with β_1 integrins, fibronectin fibrils, and actin stress fibers at extracellular matrix contact sites (data not shown). These findings indicate that β_1 integrins associate with caveolin.

To explore further the hypothesis that the recruitment of Shc by integrins was mediated by caveolin, we examined whether the association with caveolin and the recruitment of Shc required the same integrin sequences. NIH 3T3 cells expressing either the human recombinant wild-type α_1 subunit or single-chain tailless α_1 subunit were immunoprecipitated with the anti-human α_1 MAb TS2/7 or the control anti-MHC MAb W6.32, and the samples were subjected to immunoblotting with anti-caveolin antibodies. The results showed that the wild-type $\alpha_1\beta_1$ subunit and the single-chain tailless α_1 subunit associate to similar extent with the 22 kDa isoform of caveolin (Figure 5B). Prolonged exposure of the autoradiograph indicated that they both also form a complex with the 23 kDa minor isoform of caveolin. These results indicate that the association of $\alpha_1\beta_1$ with caveolin is specified by sequences contained in the membrane-proximal portion of the extracellular domain of the α subunit, its transmembrane segment, or both, i.e., the same sequences involved in the recruitment of Shc. Taken together, these findings identify caveolin as a potential mediator of the interaction between integrins and Shc.

Role of Shc in MAP Kinase Activation by Integrins

Since the recruitment of Shc is mediated by the integrin α subunit, while the activation of FAK requires the β subunit, we sought to examine whether the activation of Shc and FAK were independent phenomena. Experiments of immunoprecipitation with anti-FAK antibodies followed by immunoblotting with anti-P-Tyr antibodies indicated that ligation of single-chain tailless α_1 does

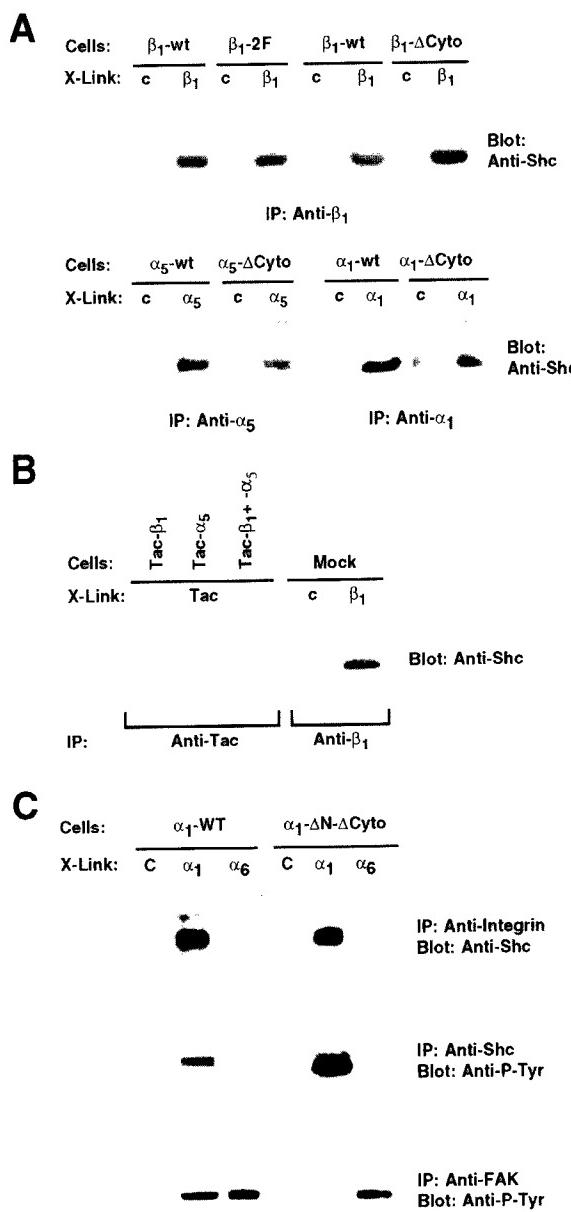


Figure 4. The Recruitment of Shc Is Specified by the Extracellular Domain of the α Subunit, Its Transmembrane Domain, or Both

(A) NIH 3T3- β_1 -WT, NIH 3T3- β_1 -2F, CHO- β_1 -WT, and CHO- β_1 - Δ Cyto cells were stimulated with W6.32 (c) or 4B4 (β_1) MAb-coated beads, immunoprecipitated with anti- β_1 MAb TS2/16, and probed by immunoblotting with anti-Shc serum. CHO- α_5 -WT, CHO- α_5 - Δ Cyto, NIH 3T3- α_1 -WT, and NIH 3T3- α_1 - Δ Cyto cells were stimulated with W6.32 (c), P1D6 (α_5), or TS2/7 (α_1) MAb-coated beads, immunoprecipitated with MAb P1D6 or TS2/7, and probed by immunoblotting with anti-Shc serum.

(B) 293-T cells were transfected with Tac- β_1 and Tac- α_5 , either separately or in combination, stimulated with anti-Tac MAb 4E3, and immunoprecipitated with the same antibody. Mock-transfected cells were stimulated with W6.32 (c) or 4B4 (β_1) MAbs and immunoprecipitated with MAb 4B4. Samples were probed with anti-Shc serum.

(C) After stimulation with W6.32 (c), TS2/7 (α_1), or GoH3 (α_6) MAbs, NIH 3T3- α_1 -WT and NIH 3T3- α_1 - Δ N- Δ Cyto cells were immunoprecipitated with the same antibodies and probed with anti-Shc serum,

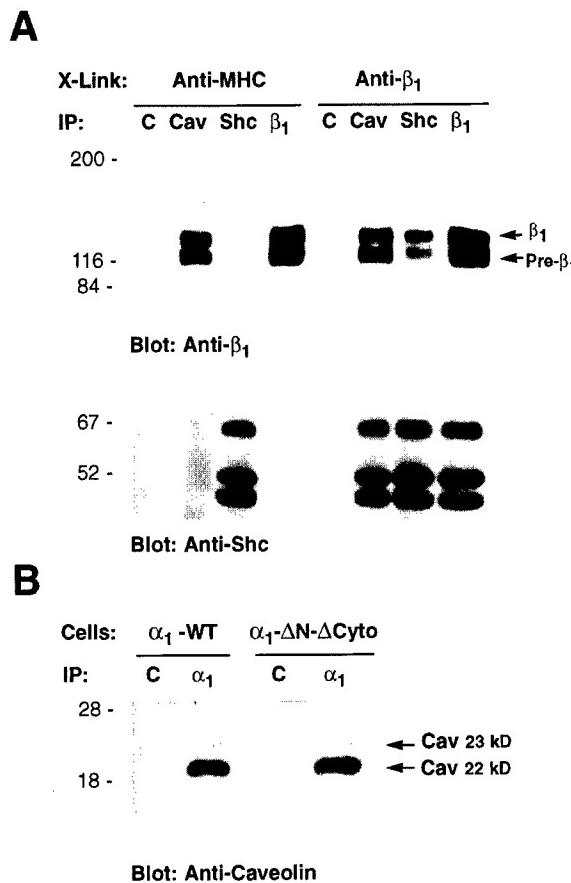


Figure 5. Association of β_1 Integrins with Caveolin

(A) A431 cells were stimulated with W6.32 (anti-MHC) or 4B4 (anti- β_1) MAbs for 10 min, immunoprecipitated with MAb W6.32 (c), C060 (Cav), clone 8 (Shc), or TS2/16 (β_1), and probed with anti- β_1 cytoplasmic domain serum or affinity-purified antibodies to Shc.

(B) NIH 3T3- α_1 -WT and NIH 3T3 α_1 -ΔN-ΔCyto cells were immunoprecipitated with W6.32 (c) or TS2/7 (α_1) MAbs followed by immunoblotting with anti-caveolin antibodies.

not induce tyrosine phosphorylation of FAK, while stimulation of wild-type $\alpha_1\beta_1$ and endogenous $\alpha_6\beta_1$ induces significant tyrosine phosphorylation of FAK (see Figure 4C). Together with the data of Figure 2, these results indicate that the single-chain tailless α_1 subunit can recruit Shc, but not activate FAK; wild-type $\alpha_6\beta_1$ can activate FAK, but not recruit Shc; and $\alpha_1\beta_1$ can both recruit Shc and activate FAK. Thus, the recruitment and tyrosine phosphorylation of Shc and the activation of FAK are separable phenomena.

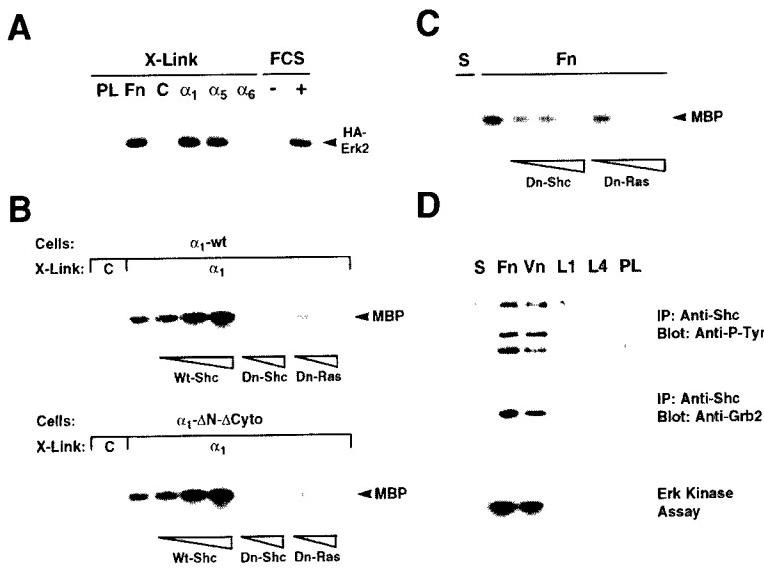
To assess the relative contribution of Shc and FAK to the activation of the Ras-mitogen-activated protein (MAP) kinase pathway by integrins, we examined the ability of various integrins to activate the MAP kinase Erk2. NIH 3T3 cells expressing the single-chain tailless

α_1 subunit were transiently transfected with a hemagglutinin (HA)-tagged Erk2 vector, stimulated with beads coated with fibronectin, various anti-integrin MAbs, or fetal calf serum (FCS), and subjected to immune complex kinase assays. As shown in Figure 6A, antibody-mediated ligation of single-chain tailless α_1 or endogenous $\alpha_5\beta_1$ activated MAP kinase to a level similar to that induced by fibronectin and serum. In contrast, cross-linking of endogenous $\alpha_6\beta_1$ did not induce this activity. Since the single-chain tailless α_1 subunit is capable of recruiting Shc, but not activating FAK, while cross-linking of $\alpha_6\beta_1$ can stimulate FAK, but does not induce association with Shc, these results suggest that Shc, and not FAK, plays a crucial role in the activation of MAP kinase by integrins.

The role of Shc in the activation of MAP kinase by integrins was further examined by testing the effect of wild-type and dominant-negative Shc (Y317F). This mutant can exert a dominant-negative effect because it is able to combine with tyrosine-phosphorylated proteins but not with Grb2 (Salcini et al., 1994; Chen et al., 1996). The introduction of wild-type Shc led to a dose-dependent increase in Erk2 activation in response to ligation of wild-type $\alpha_1\beta_1$ or single-chain tailless α_1 . Conversely, dominant-negative Shc suppressed the activation of MAP kinase in response to ligation of both molecules as effectively as dominant-negative Ras (N17) (Figure 6B). Since full activation of FAK may require cell spreading on the extracellular matrix and cytoskeletal organization, we also examined the effect of dominant-negative Shc on MAP kinase activation in NIH 3T3 cells freshly plated on fibronectin. The introduction of dominant negative Shc and dominant-negative Ras prevented the activation of Erk2 in response to cell adhesion to fibronectin (Figure 6C). We concluded that Shc plays a crucial role in the activation of the Ras-MAP kinase pathway by integrins.

The observation that only a subset of integrins can combine with Shc suggests that cell adhesion to the extracellular matrix may or may not activate Ras signaling, depending on the repertoire of integrins involved. To test this hypothesis, we examined primary human umbilical vein endothelial cells (HUVECs), because it is known that they adhere to fibronectin primarily through $\alpha_5\beta_1$ (Conforti et al., 1989), to vitronectin through $\alpha_5\beta_3$ (Cheresh, 1987), and to laminin 1 and laminin 4 through $\alpha_2\beta_1$ (Languino et al., 1989). In addition, FACS analysis indicated that they do not express the $\alpha_1\beta_1$ collagen/laminin receptor. The HUVECs were either kept in suspension or plated on dishes coated with 10 μ g/ml poly-L-lysine, fibronectin, vitronectin, laminin 1, or laminin 4. Under these conditions, the cells adhered to and spread on each extracellular matrix substratum to the same extent. As shown in Figure 6D, adhesion of HUVECs to fibronectin and vitronectin resulted in tyrosine phosphorylation of the three isoforms of Shc, association of Shc with Grb2, and activation of MAP kinase. In contrast, adhesion to laminin 1 or laminin 4 did not induce these events. These results are in accordance with the observation that $\alpha_5\beta_1$ and $\alpha_5\beta_3$, but not $\alpha_2\beta_1$, can combine with Shc and activate the Ras-MAP kinase pathway and indicate that the extracellular matrix may have selective

immunoprecipitated with anti-Shc serum and probed with a mixture of anti-P-Tyr MAbs PY20 and 4G10, or immunoprecipitated with anti-FAK polyclonal antibodies and probed with a mixture of anti-P-Tyr MAbs PY-20 and 4G10.



(C) NIH 3T3 cells were transiently transfected with 3 μ g of HA-Erk2 plasmid alone or in combination with 2.5, 5.0, and 10 μ g of dominant-negative Shc (Dn-Shc) or Ras plasmid (Dn-Ras). After starvation, the cells were either kept in suspension or plated on fibronectin-coated dishes. HA-Erk2 was immunoprecipitated and subjected to in vitro kinase assay with MBP as a substrate. Transfection efficiencies were verified as above.

(D) HUVECs were growth factor-starved and either kept in suspension or plated on dishes coated with poly-L-lysine (PL), fibronectin (Fn), vitronectin (Vn), laminin 1 (L1), or laminin 4 (L4). Lysates were immunoprecipitated with anti-Shc MAb clone 8 followed by immunoblotting with anti-P-Tyr MAb RC-20 (top) or anti-Grb2 serum (middle). HUVECs were transiently transfected with HA-Erk2 plasmid prior to plating on the various substrata. HA-Erk2 was immunoprecipitated and subjected to in vitro kinase assay with MBP as a substrate (bottom). Transfection efficiencies were verified as above.

effects on intracellular signaling depending on the integrins to which it binds.

Adhesion Mediated by Integrins Linked to Shc Promotes Transcription from the Fos Serum Response Element and Cell Cycle Progression

We next examined whether the coupling of specific integrins to Shc played a role in the control of immediate-early gene expression. Since Erk1 and Erk2 regulate transcription from the Fos serum response element (SRE) by phosphorylating the ternary complex factors Elk-1 and SAP-1 (Treisman, 1995), we examined the effect of integrin ligation on the Fos SRE. HUVECs were transiently transfected with a vector containing the Fos SRE promoter element linked to the luciferase reporter gene and plated on dishes coated with extracellular matrix proteins or poly-L-lysine. The results of the luciferase assay indicated that adhesion to fibronectin and vitronectin causes elevation of Fos SRE-dependent transcription in HUVECs, while adhesion to poly-L-lysine, laminin 1, or laminin 4 does not (Figure 7A). This result indicates that ligation of $\alpha_5\beta_1$ and $\alpha_v\beta_3$, but not $\alpha_2\beta_1$, is sufficient to promote transcription from the Fos SRE. Interestingly, treatment with basic fibroblast growth factor (bFGF) induced a significant elevation of Fos SRE activity in HUVECs plated on fibronectin and vitronectin, but caused a remarkably modest effect in cells adhering to poly-L-lysine, laminin 1, or laminin 4 (Figure 7A), suggesting that ligation of integrins linked

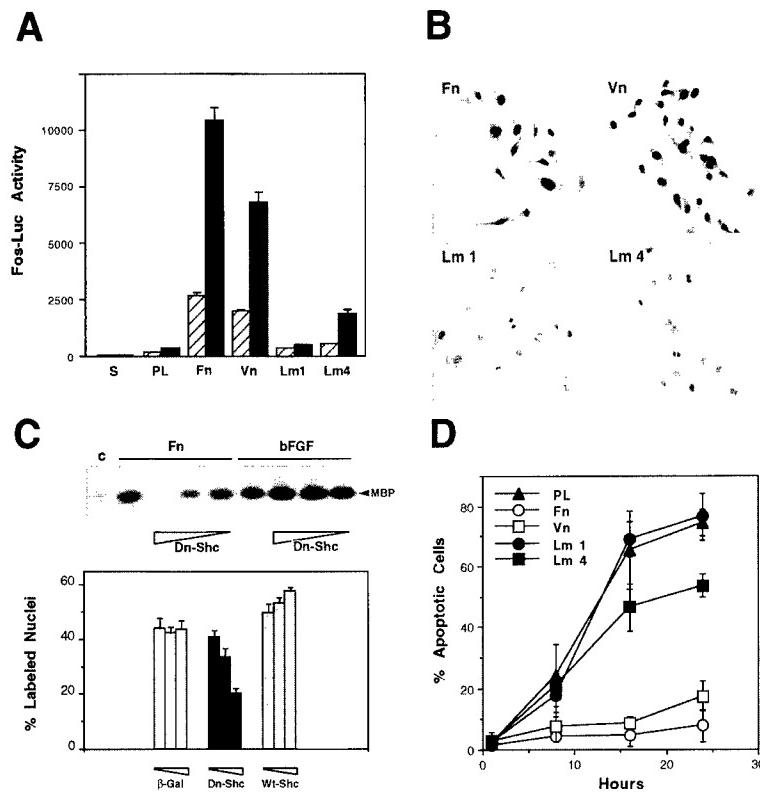
Figure 6. Role of Shc in Activation of MAP Kinase by Integrins

(A) NIH 3T3- α_1 - Δ N- Δ Cyto cells were transiently transfected with HA-tagged Erk2 plasmid, growth factor-starved, and stimulated in suspension with beads coated with poly-L-lysine (PL), fibronectin (Fn), or MAbs W6.32 (α_1), TS2/7(α_1), 5H10-27 (α_5), and GoH3 (α_6). As a control, the cells were stimulated with 10% FCS (plus) or left untreated (minus). HA-Erk2 was immunoprecipitated and subjected to in vitro kinase assay.

(B) NIH 3T3- α_1 -WT and NIH 3T3- α_1 - Δ N- Δ Cyto cells were transiently transfected with 3 μ g of Erk2-HA plasmid alone or in combination with 2.5, 5.0, and 10 μ g of wild-type Shc plasmid (Wt-Shc), 2.5 and 5.0 μ g of dominant-negative Shc plasmid (Dn-Shc), and 2.5 and 5.0 μ g of dominant-negative Ras plasmid (Dn-Ras). The cells were stimulated with MAbs W6.32 (c) or TS2/7 (α_1). Immunoprecipitated HA-Erk2 was subjected to in vitro kinase assay with MBP as a substrate. Transfection efficiencies were verified by immunoblotting aliquots of total proteins with anti-HA antibodies.

to Shc, such as $\alpha_5\beta_1$ or $\alpha_v\beta_3$, is required for induction of Fos SRE-dependent transcription in cells exposed to mitogenic growth factors.

To examine whether the integrins linked to Shc played a role in cell cycle progression, HUVECs were synchronized in G0 by growth factor starvation and then plated in presence of mitogens on plastic wells coated with extracellular matrix proteins or poly-L-lysine. Entry into the S phase was examined by 5'-bromo-2'-deoxy-uridine (BrdU) incorporation and anti-BrdU staining. The large majority of HUVECs adhering to fibronectin (96.9% \pm 2.7%) and vitronectin (96.2% \pm 2.1%) entered the S phase during the 24 hr of the assay. In contrast, only a modest percentage of cells plated on laminin 1 (11.8% \pm 4.4%), laminin 4 (21.2% \pm 9%), or poly-L-lysine (0.8% \pm 1.3%) entered into S phase under the same conditions (Figure 7B). Since this percentage did not increase over an additional 24-hr period, it is unlikely that adhesion to poly-L-lysine or laminins simply delays entry into S phase. In addition, because the HUVECs acquired and maintained a well-spread morphology on laminins (Figure 7B), their inability to enter into S phase on these substrata is not the result of insufficient spreading. Interestingly, if the endothelial cells were plated on poly-L-lysine or laminins in the presence of 10% FCS, which contains fibronectin and vitronectin, they progressed normally through G1 and entered the S phase. The results of these experiments indicate that attachment and spreading on the extracellular matrix are not sufficient for progression of primary cells through G1 in



fied by immunoblotting with anti-HA MAb. Immunoprecipitated HA-Erk2 was subjected to in vitro kinase assay with MBP as a substrate (top). HUVECs were transiently transfected with 10, 5, and 2.5 μ g of plasmids encoding HA-tagged β -galactosidase (β -Gal), FLAG-tagged dominant negative Shc (Dn-Shc), or FLAG-tagged wild-type Shc (WT-Shc). The percentage of transfected cells entering into S was determined as described in Experimental Procedures. The diagram shows the mean value and standard deviation from triplicate samples (bottom). (D) G0-synchronized HUVECs were plated on wells coated with 10 μ g/ml poly-L-lysine (PL), fibronectin (Fn), laminin 1 (Lm1), or laminin 4 (Lm4). Adherent cells were incubated in defined medium for the indicated times. At each time point, attached and unattached cells were combined and stained in suspension with Hoechst dye. The percentage of apoptotic nuclei was determined by scoring at least 500 cells from five different microscopic fields. Diagram indicates the mean and standard deviation from triplicate samples.

response to mitogens, but that this process requires ligation of integrins, such as $\alpha_5\beta_1$ and $\alpha_v\beta_3$, which are coupled to Ras signaling by Shc.

To examine further the role of integrin signaling in cell proliferation, we examined the effect of dominant-negative or wild-type Shc on HUVEC progression through G1. Varying concentrations of vectors encoding FLAG-tagged dominant-negative or wild-type Shc were transfected in HUVECs. After growth factor starvation and restimulation with mitogens, the entry into S phase of cells expressing FLAG-tagged Shc molecules was monitored by double immunofluorescent staining with anti-FLAG and anti-BrdU antibodies. As a control, HUVECs were transfected with varying doses of a vector encoding HA-tagged β -galactosidase, and the entry into S phase of these cells was evaluated by double immunofluorescent staining with anti-HA and anti-BrdU antibodies. As shown in Figure 7C (bottom), the introduction of dominant-negative Shc caused a dose-dependent inhibition of HUVEC entry into S phase. Conversely, wild-type Shc accelerated entry into S, although to a limited extent. The inhibition of cell cycle progression caused by dominant-negative Shc was significant but incomplete, perhaps because transient transfection does not allow

high-level expression of the inhibitory molecule over the relatively prolonged duration of G1. It is unlikely that dominant-negative Shc interferes with mitogen stimulation of HUVECs, because it affected the activation of Erk2 by fibronectin, but not by bFGF (Figure 7C, top). These results are consistent with the observation that the FGF receptor 1 is linked to Ras by both Shc-dependent and Shc-independent mechanisms (Mohammadi et al., 1996) and support the notion that the coupling of integrins to Ras signaling mediated by Shc regulates cell cycle progression.

Adhesion Mediated by Integrins Not Linked to Shc Results in Apoptotic Death

A large fraction of endothelial cells, which initially adhered on poly-L-lysine or laminins, subsequently lost phase density and detached, despite being exposed to optimal concentrations of growth factors. We therefore examined whether ligation of integrins linked to Shc protected endothelial cells from apoptosis. HUVECs were plated at low density in the presence of mitogens on dishes coated with extracellular matrix molecules or poly-L-lysine. At various times after plating, adherent

Figure 7. Control of Fos-SRE-Dependent Transcription, Cell Survival, and Cell Cycle Progression by the Extracellular Matrix

(A) HUVECs were transiently transfected with Fos-SRE-Luc plasmid. After growth factor starvation, the cells were either kept in suspension (S) or plated onto dishes coated with 10 μ g/ml poly-L-lysine (PL), fibronectin (Fn), laminin 1 (Lm1), or laminin 4 (Lm4) for 20 min. The cells were then either left untreated (hatched bars) or exposed to 10 ng/ml bFGF and 1 μ g/ml heparin (closed bars) for 5 min. Cell lysates containing equal amounts of total proteins were subjected to luciferase assay. Values are expressed in arbitrary units.

(B) HUVECs were growth factor-starved and plated for 24 hr in defined medium containing 10 μ M BrdU onto wells coated with 10 μ g/ml fibronectin (Fn), vitronectin (Vn), laminin 1 (Lm1), or laminin 4 (Lm4). After immunostaining with anti-BrdU monoclonal antibodies and alkaline phosphatase-conjugated secondary antibodies, the cells were lightly counterstained with hematoxylin.

(C) HUVECs were transiently transfected with 3 μ g of HA-Erk2 plasmid alone or in combination with 10, 5, and 2.5 μ g of plasmid encoding dominant-negative Shc (Dn-Shc). After growth factor starvation, the cells were detached and kept in suspension (c) or plated on fibronectin-coated dishes (Fn), or they were not detached but were treated with 25 ng/ml bFGF and 1 μ g/ml heparin for 5 min (bFGF). Transfection efficiencies were veri-

and floating cells were removed, combined, and examined for features of apoptotic death by staining with Hoechst dye. As shown in Figure 7D, only a small number of HUVECs plated on fibronectin or vitronectin underwent apoptosis during the 24 hr of the assay. In contrast, the majority of cells plated on poly-L-lysine or laminins became apoptotic by the end of the assay. Direct staining of cells adhering to poly-L-lysine or laminins revealed that most of them had become apoptotic by 12 hr, suggesting that detachment follows apoptosis and not vice versa. These findings suggest that ligation of integrins linked to Shc is required for endothelial cell survival.

Discussion

The results of this study indicate that a subset of β_1 integrins and $\alpha_v\beta_3$ are linked to Ras signaling and immediate-early gene expression by the adaptor protein Shc. Ligation of integrins linked to Shc enables primary endothelial cells to progress through G1 in response to mitogens, whereas ligation of other integrins, under the same conditions, results in exit from the cell cycle. On the basis of these findings and the ability of dominant-negative Shc to inhibit cell cycle progression without affecting mitogen signaling, we propose that the association of specific integrins with Shc controls cell cycle progression in response to the extracellular matrix. Since exit from the cell cycle is a prerequisite for cell differentiation, our results may also explain why interaction with the extracellular matrix in some settings promotes differentiation.

Shc is a SH2-PTB domain adaptor protein that links various tyrosine-phosphorylated signal transducers to Ras (Pawson, 1995). We have previously shown that ligation of the $\alpha_6\beta_4$ integrin, which is associated with a tyrosine kinase, causes phosphorylation of the β_4 tail and direct recruitment of Shc (Mainiero et al., 1995). In this study, we provide evidence that specific β_1 integrins and $\alpha_v\beta_3$ also combine with Shc, but by a distinct and novel mechanism. In this case, the recruitment of Shc is mediated by the membrane-proximal portion of the extracellular domain of the integrin α subunit, its transmembrane segment, or both. Coimmunoprecipitation experiments revealed that this region of the α subunit interacts constitutively with caveolin, a two-pass transmembrane adaptor involved in linking a variety of cell-surface receptors to intracellular signaling pathways (Lisanti et al., 1994), and indicated that Shc associates with caveolin in response to integrin ligation. Although a definitive demonstration that the recruitment of Shc to β_1 integrins and $\alpha_v\beta_3$ is mediated by caveolin will require further biochemical and mutational analysis, this model is intriguing, because caveolin is phosphorylated on tyrosine in cells transformed by v-Src (Glenney and Soppet, 1992) and has also been shown to interact with c-Fyn (Corley Mastick et al., 1995). Thus, caveolin may provide both the adaptor and the tyrosine kinase necessary for the recruitment and tyrosine phosphorylation of Shc in response to integrin ligation.

There are several reasons to believe that the association of specific integrins with Shc mediates activation of the MAP kinase pathway and transcription of Fos in

response to the extracellular matrix. First, ligation of $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$, which are linked to Shc, results in MAP kinase activation, but ligation of other integrins does not produce this effect, despite stimulating FAK. Second, cross-linking of the single-chain tailless α_1 subunit causes recruitment and tyrosine phosphorylation of Shc and activation of MAP kinase without inducing FAK activation. Third, a dominant-negative version of Shc suppresses MAP kinase activation in response to integrin ligation. Fourth, while adhesion of endothelial cells to fibronectin and vitronectin, which is mediated by the Shc-linked $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins, activates MAP kinase and induces transcription from the Fos SRE, their interaction with laminin 1 and laminin 4, which is mediated by $\alpha_2\beta_1$, does not cause these effects. Taken together, these results indicate that Shc plays a crucial role in the activation of the Ras-MAP kinase pathway and Fos gene expression in response to the extracellular matrix.

Our findings suggest that the association of specific integrins with Shc regulates cell cycle progression in normal cells. In fact, engagement of integrins linked to Shc activates SRE-dependent transcription and promotes progression through G1 in response to growth factors. In contrast, ligation of other integrins results in cell cycle arrest even in the presence of otherwise mitogenic concentrations of growth factors. Normal cells require a signal from the extracellular matrix in order to proliferate. Recent studies have indicated that cell adhesion is necessary for the induction of cyclin D₁ and activation of the cyclin E-cdk2 complex in early to mid-G1 (Fang et al., 1996; Zhu et al., 1996), suggesting that mitogen- and cell adhesion-dependent signals are integrated prior to the induction of cyclin D₁. In accordance with this hypothesis, our findings suggest that integrin- and growth factor-dependent signals converge on Ras.

It is likely that a simultaneous stimulation of Ras by integrins and growth factor receptors is needed to reach the threshold level of MAP kinase activation required for optimal transcription of immediate-early response genes. Since mitogenic growth factors induce a rapid and short-lived stimulation of MAP kinase, while cell adhesion produces a long-lasting activation of the enzyme (Zhu and Assoian, 1995), the two stimuli may also cooperate kinetically. This model is consistent with the observation that overexpression of Shc (Pelicci et al., 1992) and constitutive activation of MAP kinase (Cowley et al., 1994) lead to anchorage-independent cell growth. Furthermore, since most dominant oncogenes transform cells by activating the Ras-MAP kinase pathway, it also explains why most transformed cells display anchorage-independent growth.

Recent studies have revealed that normal cells denied anchorage to the extracellular matrix undergo apoptosis (Ruoslathi and Reed, 1994). The findings of this study suggest that the ability of the extracellular matrix to promote cell survival is mediated by the coupling of specific integrins to Shc. Since inhibition of MAP kinase or activation of Jun kinase cause apoptosis (Xia et al., 1995), it is possible that the integrins that combine with Shc promote cell survival by elevating the activity of MAP kinase, thereby increasing the ratio of MAP kinase

to Jun kinase activity. The anti-apoptotic function of integrins linked to Shc is consistent with the results of previous studies. For example, antibodies and synthetic peptides, which interfere with the adhesive function of $\alpha_v\beta_3$, induce endothelial cell apoptosis and thereby blunt angiogenesis *in vivo* (Brooks et al., 1994). We suggest, on the basis of our results, that these reagents do not trigger intracellular signals that lead to apoptosis, but rather prevent the activation of Shc signaling caused by $\alpha_v\beta_3$ ligation. The ability of fibronectin to protect CHO cells from apoptosis in response to serum withdrawal (Zhang et al., 1995) and that of basement membrane components, but not type I collagen, to promote survival of breast epithelial cells (Pullan et al., 1996) may also depend on the engagement of specific integrins linked to Shc.

We have observed that intercellular contact can rescue primary endothelial cells plated on laminins from apoptotic death (unpublished data). It has been reported that cell-to-cell contact exerts a similar protective effect in primary breast epithelial cells (Pullan et al., 1996). Although the mechanism by which physical contact between cells protects primary cells from apoptosis is not known, these observations suggest that multiple positional signals contribute to cell survival *in vivo*. It can be envisioned that *in vivo* these signals promote the survival of those cells that have, as part of their natural life cycle, lost contact with a matrix capable of activating the Shc pathway. This mechanism may ensure that only cells displaced from their natural environment are eliminated.

What is the fate of those cells that survive despite lacking the Shc signal? The observation that extracellular matrix recognition by integrins that fail to activate Shc results in cell cycle exit even in the presence of mitogens suggests that these cells may be induced to differentiate. It is known that withdrawal from the cell cycle is a prerequisite for differentiation, and several mechanisms ensure that proliferation and differentiation are mutually exclusive. For example, active cyclin D₁-cdk complexes suppress MyoD function in proliferating myoblasts, thereby preventing the expression of muscle-specific genes (Skapek et al., 1995). Conversely, MyoD may maintain the G0 arrest of differentiated skeletal muscle by acting on Rb (Gu et al., 1993). These observations suggest that those integrins that do not activate the Shc pathway may promote differentiation primarily because they do not induce cyclin D₁-cdk levels sufficient to block the function of transcription factors involved in differentiation.

The existence of two classes of integrins with distinct signaling properties explains a number of previous observations. In various cell types, interaction with fibronectin promotes proliferation and inhibits differentiation, while adhesion to laminin promotes cell cycle withdrawal and morphological and functional differentiation. For example, endothelial cells plated on fibronectin proliferate, but on a laminin-rich matrix they cease growing and rapidly form capillary-like structures (Kubota et al., 1988). Similarly, myoblasts proliferate on fibronectin, but fuse to form myotubes on laminin (von der Mark and Ocalan, 1989). These two opposing functions have been linked to the expression of $\alpha_5\beta_1$ and $\alpha_6\beta_1$, respectively

(Sastry et al., 1996). Furthermore, recent studies have indicated that the binding of fibronectin to $\alpha_5\beta_1$, but not $\alpha_4\beta_1$, results in induction of the collagenase gene in synovial fibroblasts, suggesting that the same matrix molecule may or may not induce gene expression depending on the integrin to which it binds (Huhtala et al., 1995). Taken together, our findings suggest that the differential responses of a given cell type to extracellular matrices of different composition and of different cell types to the same extracellular matrix protein may all depend on the ability of a class of integrins to activate Shc signaling.

Experimental Procedures

Antibodies and Extracellular Matrix Molecules

The MAbs TS2/7, FW-14-14-15, and TS2/16 were obtained from the American Type Culture Collection, P1E6, P1B5, P1D6, and P4H9 from GIBCO BRL, 5H10-27 from Pharmingen, GoH3 from Immunotech, 4B4 from Coulter, 4E3 and 12CA5 from Boehringer, M2 from Eastman-Kodak, 4G10 from Upstate Biotechnology, Incorporated, and PY20, RC-20, clone 8, and C060 from Transduction Laboratories. The anti- β , cytoplasmic domain serum and serum 410, which reacts predominantly with the 52 kDa isoform of Shc, were described (Giancotti and Ruoslahti, 1990; Mainiero et al., 1995). The MAbs AIIB2 and BIE5 were provided by C. Damsky, LM609 by D. Cheresh, and 135-13C by S. Kennel. The anti-P-Tyr serum 72 and anti-Grb2 MAb EL-6 were generated in the laboratory of J. Schlessinger. Affinity-purified rabbit antibodies to Shc and caveolin were from Upstate Biotechnology and Transduction Laboratories, respectively. Anti-Grb2 and anti-Erk2 sera were from Santa Cruz Biotechnology. The anti-FAK peptide serum was generated in the laboratory of G. Tarone. Human fibronectin, vitronectin, laminin 4 (placental meroisin; Spinardi et al., 1995), and mouse laminin 1 were purchased from GIBCO BRL.

Cell Lines, Constructs, and Transfections

CHO cells expressing β_1 -WT and β_1 -ΔCyto were generated in the laboratory of G. Tarone (University of Torino, Turin, Italy). CHO cells expressing α_5 -WT or α_5 -ΔCyto and NIH 3T3 cells expressing α_1 -WT, α_1 -ΔCyto, or α_1 -ΔN-ΔCyto were previously described (Bauer et al., 1993; Briesewitz et al., 1993; Kern et al., 1994). NIH 3T3 cells expressing β_1 -WT and β_1 -2F were generated as previously described (Giancotti et al., 1994). Transient transfection of vectors encoding Tac- α_5 Cyto and Tac- β_1 Cyto (LaFlamme et al., 1992) in 293-T cells was also done as described (Giancotti et al., 1994). The Fos(SRE)-Luc reporter plasmid (from J. Schlessinger) and vectors encoding HA-tagged Erk2, HA-tagged β -galactosidase, FLAG-tagged and untagged wild-type or dominant-negative p52^{shc} (Y317F), and dominant negative Ras (N17) (from E. Scolnik, New York University School of Medicine) were transiently transfected in NIH 3T3 cells by the lipofectamine method and in HUVECs by the lipofectin method (GIBCO BRL).

Biochemical Methods

After ligation of integrins (Mainiero et al., 1995), cells were extracted and subjected to immunoprecipitation followed by immunoblotting or kinase assay. To immunoprecipitate Shc, integrins, caveolin, and FAK, cells were extracted in 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100. To immunoprecipitate Erk2, cells were extracted with 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM EDTA. Immunoprecipitation, immunoblotting, and GST fusion protein binding experiments were performed as previously described (Mainiero et al., 1995). Erk2 immune-complex kinase assays were performed in 50 mM Tris (pH 7.5), 10 mM MgCl₂ containing 5 μ Ci of [γ -³²P]ATP (4500 Ci/mmol, ICN Biomedicals, Incorporation) and 2.5 μ g of MHC-binding protein (MBP). Ras-GTP loading assays were performed as described (Gale et al., 1993). To measure SRE-dependent transcription, HUVECs were transiently transfected with Fos-SRE-Luc. After growth factor starvation, the cells were detached, plated on dishes coated with 10 μ g/ml poly-L-lysine, fibronectin, vitronectin, laminin

1, or laminin 4, and then subjected to luciferase assay. At this coating concentration, all extracellular matrix proteins promoted similar levels of adhesion and spreading in HUVECs.

Measurement of Cell Cycle Progression and Apoptosis

To monitor progression through G1 and entry into S phase, HUVECs were synchronized in G0 by growth factor starvation, detached, and plated at low density on microtiter plates or glass coverslips coated with poly-L-lysine or extracellular matrix molecules. After 24 hr of incubation in defined medium (M199 supplemented with 25 ng/ml bFGF, 1 µg/ml heparin, 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 0.625 ng/ml selenous acid, 1.25 mg/ml BSA, and 5.35 µg/ml linoleic acid) containing 10 µM BrdU, cells were stained with anti-BrdU MAb and AP-conjugated anti-mouse IgGs (Boehringer).

To test the effect of dominant-negative and wild-type Shc on cell cycle progression, HUVECs were transiently transfected with vectors encoding FLAG-tagged dominant-negative Shc, FLAG-tagged wild-type Shc, or the control protein HA-β-galactosidase. After incubation in complete medium for 8 hr, the cells were starved for 24 hr and then incubated in defined medium containing 10 µM BrdU for 24 hr. Entry into S phase of transfected cells was monitored by double immunofluorescent staining with anti-FLAG MAb M2 or anti-HA MAb 12CA5 followed by Texas red-conjugated anti-mouse IgGs and FITC-conjugated anti-BrdU antibodies.

To measure apoptosis, G0-synchronized HUVECs were plated on wells or coverslips coated with poly-L-lysine or extracellular matrix proteins and incubated in defined medium for the indicated times. Attached and unattached cells were combined and stained in suspension with Hoechst dye.

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The coupling of $\alpha_6\beta_4$ integrin to Ras–MAP kinase pathways mediated by Shc controls keratinocyte proliferation

Fabrizio Mainiero^{1,2}, Chiara Murgia^{1,3},
Kishore K.Wary^{1,3}, Anna Maria Curatola^{1,4},
Angela Pepe^{1,3}, Miroslav Blumemberg⁵,
John K.Westwick⁶, Channing J.Der⁶ and
Filippo G.Giancotti^{1,3,7}

¹Department of Pathology and ⁵Department of Dermatology, Kaplan Cancer Center, New York University School of Medicine, New York, NY 10016 and ⁶Department of Pharmacology, Linenbergs Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

²Present address: Dipartimento di Medicina Sperimentale e Patologia, Università La Sapienza, Viale Regina Elena 324, 00161 Roma, Italy

³Present address: Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA

⁴Present address: Department of Pediatrics, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA

⁷Corresponding author
e-mail: F-GIANCOTTI@ski.mskcc.org

The signaling pathways linking integrins to nuclear events are incompletely understood. We have examined intracellular signaling by the $\alpha_6\beta_4$ integrin, a laminin receptor expressed in basal keratinocytes and other cells. Ligation of $\alpha_6\beta_4$ in primary human keratinocytes caused tyrosine phosphorylation of Shc, recruitment of Grb2, activation of Ras and stimulation of the MAP kinases Erk and Jnk. In contrast, ligation of the laminin- and collagen-binding integrins $\alpha_3\beta_1$ and $\alpha_2\beta_1$ did not cause these events. While the stimulation of Erk by $\alpha_6\beta_4$ was suppressed by dominant-negative Shc, Ras and RhoA, the activation of Jnk was inhibited by dominant-negative Ras and Rac1 and by the phosphoinositide 3-kinase inhibitor Wortmannin. Adhesion mediated by $\alpha_6\beta_4$ induced transcription from the Fos serum response element and promoted cell cycle progression in response to mitogens. In contrast, $\alpha_3\beta_1$ - and $\alpha_2\beta_1$ -dependent adhesion did not induce these events. These findings suggest that the coupling of $\alpha_6\beta_4$ integrin to the control of cell cycle progression mediated by Shc regulates the proliferation of basal keratinocytes and possibly other cells which are in contact with the basement membrane *in vivo*.

Keywords: integrins/keratinocytes/MAP kinase/Ras/Shc

Introduction

In addition to promoting cell adhesion and contributing to the organization of tissues and organs, basement membranes exert complex and often divergent effects on the survival, proliferation and differentiation of epithelial cells (Adams and Watt, 1993; Lin and Bissel, 1993). The influences of basement membranes on epithelial cells are likely to be mediated by the ability of laminin-binding

integrins to induce intracellular signaling (Giancotti and Mainiero, 1994; Clark and Brugge, 1995; Schwartz *et al.*, 1995), but the mechanisms involved are incompletely understood.

To elucidate the effects of laminins on epithelial cells, we have focused on the $\alpha_6\beta_4$ integrin. Cell adhesion assays with $\alpha_6\beta_4$ -transfected K562 cells and radioligand binding studies with purified recombinant $\alpha_6\beta_4$ have indicated that this integrin is a receptor for various laminin isoforms and binds with the highest apparent affinity to laminins 5 and 4 (Niessen *et al.*, 1994; Spinardi *et al.*, 1995). In accordance with its role as a basement membrane receptor, $\alpha_6\beta_4$ is expressed in epithelial cells (Kajiji *et al.*, 1989), Schwann cells (Sonnenberg *et al.*, 1990; Einheber *et al.*, 1993) and a subset of endothelial cells (Kennel *et al.*, 1992; Klein *et al.*, 1993) and thymocytes (Wadsworth *et al.*, 1992).

The $\alpha_6\beta_4$ integrin has a distinctive structure and subcellular localization. The large cytoplasmic domain of the β_4 subunit, which is characterized by two pairs of type III fibronectin (Fn)-like domains separated by a 142 amino acid sequence (connecting segment), does not contain any region of homology with the cytoplasmic domains of other known integrin β subunits (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990). Furthermore, while β_1 and α_v integrins are concentrated in focal adhesions and linked to the actin filament system, the $\alpha_6\beta_4$ integrin is found in hemidesmosomes both *in vivo* and in cultured cells (Carter *et al.*, 1990a; Stepp *et al.*, 1990). These observations suggest that the unique cytoplasmic domain of β_4 interacts with cytoskeletal elements of hemidesmosomes, thereby linking $\alpha_6\beta_4$ to the keratin filament system.

Our previous studies have indicated that the association of $\alpha_6\beta_4$ with the hemidesmosomal cytoskeleton is mediated by the cytoplasmic domain of β_4 and specifically by a region which includes the first pair of type III Fn-like repeats and the connecting segment (Spinardi *et al.*, 1993). In accordance with the hypothesis that $\alpha_6\beta_4$ plays a crucial role in the assembly of hemidesmosomes and their linkage to the keratin filament system, we have observed that the introduction of a truncated tail-less β_4 subunit into cells possessing endogenous $\alpha_6\beta_4$ integrins and hemidesmosomes results in a dominant-negative effect on hemidesmosome assembly (Spinardi *et al.*, 1995). Since the tail-less integrin binds efficiently to extracellular ligand, its dominant-negative effect is likely to result from its ability to co-cluster with the endogenous wild-type receptor and block a signal necessary for hemidesmosome assembly. In accordance with this hypothesis, recent studies have revealed that $\alpha_6\beta_4$ is associated with an intracellular tyrosine kinase. Mutagenesis experiments have provided evidence that the phosphorylation of a tyrosine activation motif (TAM) located in the connecting segment controls the association of $\alpha_6\beta_4$ with the hemidesmosomal

cytoskeleton, presumably via the recruitment of a signaling molecule containing two tandem Src homology 2 (SH2) domains (Mainiero *et al.*, 1995).

In the epidermis and other stratified epithelia, the expression of $\alpha_6\beta_4$ is restricted to the basal cell layer which contains cells endowed with proliferative capacity (Kajiji *et al.*, 1989). It is known that keratinocytes exit the cell cycle and begin their differentiation program when they detach from the basement membrane to migrate to the upper epidermal layers (Hall and Watt, 1989). In fact, this process can be replicated *in vitro* by depriving cultured keratinocytes of anchorage to their endogenously produced extracellular matrix (Green, 1977), which is particularly rich in laminin 5 (Carter *et al.*, 1991; Rouselle *et al.*, 1991). Furthermore, squamous carcinoma cells endowed with high proliferative potential often express elevated levels of $\alpha_6\beta_4$ (Kimmel and Carey, 1986; Wolf *et al.*, 1990). Finally, the basal keratinocytes of β_4 knock-out mice display signs of degeneration even in areas of epidermis where no significant detachment from the basement membrane is observed (Dowling *et al.*, 1996). These observations suggest that $\alpha_6\beta_4$ may provide epithelial cells with a signal important for cell survival and cell cycle progression.

What is the mechanism by which the $\alpha_6\beta_4$ integrin transduces biochemical signals capable of affecting cell proliferation? Immunoprecipitation and GST fusion protein binding experiments have indicated that ligation of $\alpha_6\beta_4$ results in the association of the adaptor protein Shc with tyrosine-phosphorylated β_4 . Shc is then phosphorylated on tyrosine residues, presumably by the integrin-associated kinase, and combines with the other adaptor protein Grb2 (Mainiero *et al.*, 1995). These observations raise two key questions. First, what are the biochemical consequences of the recruitment of Shc and Grb2 to $\alpha_6\beta_4$? Second, what is the biological significance of $\alpha_6\beta_4$ signaling in epithelial cells?

In this study, we provide evidence that the $\alpha_6\beta_4$ integrin stimulates the Ras-Erk and Rac-Jnk mitogen-activated protein kinase (MAP kinase) signaling pathways via Shc and thereby controls immediate-early gene expression and keratinocyte proliferation in response to laminin.

Results

Ligation of $\alpha_6\beta_4$ causes activation of the Ras-Erk signaling pathway

To examine the intracellular signaling pathways activated by the $\alpha_6\beta_4$ integrin in a physiologically relevant cellular context, we elected to use primary human keratinocytes. These cells express high levels of $\alpha_6\beta_4$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ and adhere to laminin 5 through $\alpha_6\beta_4$ and $\alpha_3\beta_1$ (Xia *et al.*, 1996) and to collagen I through $\alpha_2\beta_1$ (Carter *et al.*, 1990b). Incubation of suspended keratinocytes with polystyrene beads coated with the anti- β_4 monoclonal antibody (Mab) 3E1 as well as adhesion to laminin 5-coated dishes caused tyrosine phosphorylation of the 52 and 46 kDa mol. wt isoforms of Shc (the 66 kDa isoform of Shc is expressed at very low levels in keratinocytes) and thereby recruitment of Grb2 (Figure 1A). No significant tyrosine phosphorylation of Shc and recruitment of Grb2 was observed in keratinocytes treated with beads coated with the anti- $\alpha_3\beta_1$ Mab P1B5 (not shown) or the control anti-MHC Mab

W6.32 and in keratinocytes adhering to collagen I or poly-L-lysine-coated dishes (Figure 1A). The inability of collagen I to induce tyrosine phosphorylation of Shc and recruitment of Grb2 was not a consequence of insufficient adhesion because the keratinocytes spread equally well on laminin 5 and collagen I under our experimental conditions. Furthermore, we observed that antibody-mediated cross-linking of $\alpha_2\beta_1$ does not induce tyrosine phosphorylation of Shc and recruitment of Grb2 (not shown). These findings, which are consistent with previous results (Mainiero *et al.*, 1995; Wary *et al.*, 1996), indicate that ligation of $\alpha_6\beta_4$, but not $\alpha_2\beta_1$ and $\alpha_3\beta_1$, can promote signaling mediated by Shc in primary keratinocytes.

We next examined the role of cytoplasmic domain of β_4 in the recruitment and tyrosine phosphorylation of Shc. Rat bladder 804G cells expressing either a recombinant full-length human β_4 subunit (clone A) or a tail-less version (clone L) were cross-linked in suspension with the anti- β_4 Mab 3E1 or the control anti-MHC Mab W6.32. Immunoprecipitation with the 3E1 Mab followed by immunoblotting with anti-Shc antibodies revealed that the recruitment of all three isoforms of Shc by $\alpha_6\beta_4$ requires the cytoplasmic domain of the β_4 subunit (Figure 1B). To examine if recruitment to $\alpha_6\beta_4$ was a prerequisite for tyrosine phosphorylation of Shc, clone A and clone L were either cross-linked in suspension with the 3E1 or the W6.32 Mab or plated onto dishes coated with the same antibodies. Immunoprecipitation with anti-Shc antibodies followed by immunoblotting with anti-P-Tyr antibodies indicated that ligation of wild-type $\alpha_6\beta_4$ induces tyrosine phosphorylation of the 52 kDa isoform of Shc (the major isoform recruited to activated $\alpha_6\beta_4$ in these cells). In contrast, ligation of tail-less $\alpha_6\beta_4$ did not cause efficient tyrosine phosphorylation of Shc (Figure 1C). These results are consistent with the hypothesis that the recruitment of Shc to activated $\alpha_6\beta_4$ is mediated by tyrosine phosphorylation of the cytoplasmic domain of β_4 . They also suggest that this event is necessary for subsequent tyrosine phosphorylation of the adaptor protein. Since in a number of experiments tail-less $\alpha_6\beta_4$ was able to induce a modest level of tyrosine phosphorylation of Shc, it is possible that an additional indirect mechanism contributes to the activation of Shc by $\alpha_6\beta_4$.

Since Grb2 is stably associated with the Ras-GTP exchange factor mSOS, the recruitment of Grb2 to the plasma membrane mediated by Shc is likely to bring mSOS in close proximity to its target Ras (Schlessinger, 1994). Ras-GTP loading experiments were therefore performed to examine if ligation of $\alpha_6\beta_4$ resulted in activation of Ras. After growth factor starvation and *in vivo* labeling with [³²P]orthophosphate, primary human keratinocytes were detached and either incubated in suspension with anti- β_4 or anti-MHC beads or replated on dishes coated with laminin 5 or anti- β_4 Mab. As a control, adherent keratinocytes were either left untreated or were stimulated with epidermal growth factor (EGF). As shown in Figure 2, chromatographic analysis of nucleotides bound to Ras indicated that adhesion to laminin 5- or anti- β_4 Mab-coated dishes results in an accumulation of GTP on Ras comparable with that caused by EGF. In suspended cells, however, antibody-mediated ligation of $\alpha_6\beta_4$ did not cause activation of Ras (Figure 2). The results of this experiment indicate that $\alpha_6\beta_4$ -mediated cell adhesion causes activation

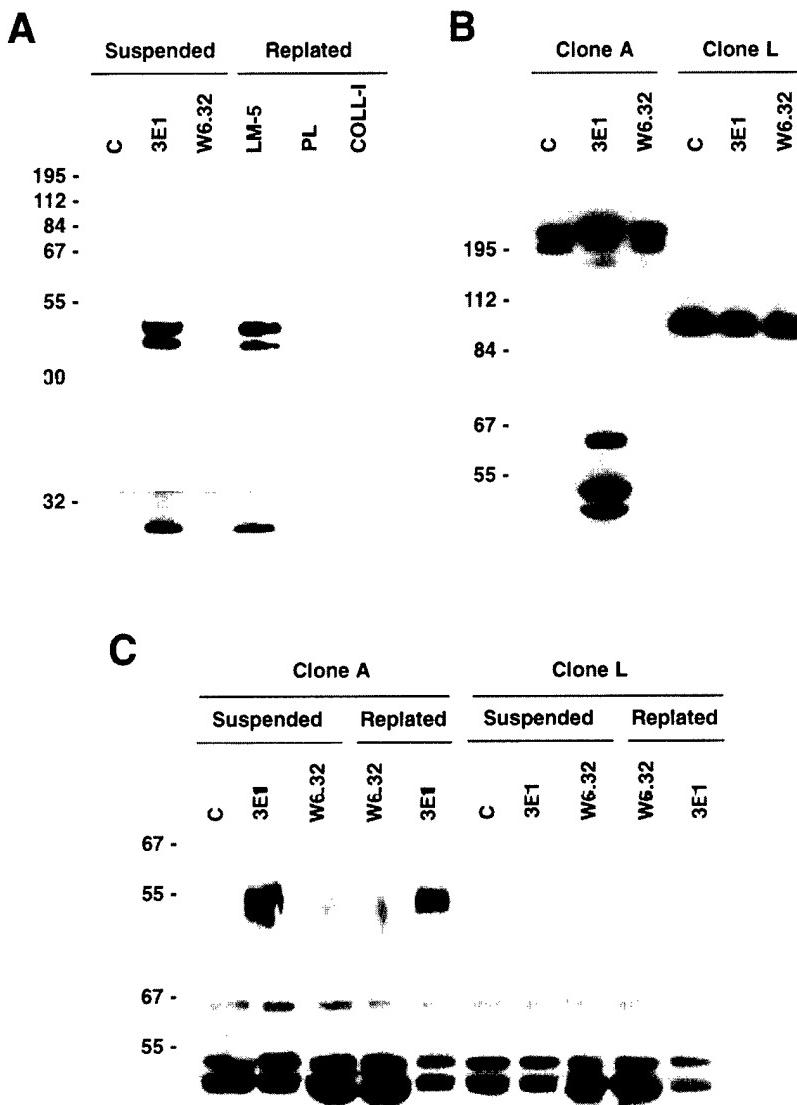


Fig. 1. Ligation of the $\alpha_6\beta_4$ integrin induces recruitment of Shc, tyrosine phosphorylation of Shc and association of Shc with Grb2. (A) Tyrosine phosphorylation of Shc and association of Shc with Grb2 in response to $\alpha_6\beta_4$ ligation. After growth factor starvation, primary human keratinocytes were detached and either incubated in suspension with medium alone (C), anti- β_4 beads (3E1) or anti-MHC beads (W6.32) for 20 min or plated on dishes coated with laminin 5 (LM-5), poly-L-lysine (PL) or collagen I (COLL-I) for 30 min. Equal amounts of total proteins were immunoprecipitated with anti-Shc Mab. The samples were probed by immunoblotting with HRP-conjugated anti-P-Tyr Mab RC20 (top panel) or polyclonal anti-Grb2 antibodies (bottom panel). (B) The recruitment of Shc to activated $\alpha_6\beta_4$ is mediated by the cytoplasmic domain of β_4 . Clone A and clone L cells were serum starved and incubated in suspension with medium alone (C), with anti- β_4 beads (3E1) or anti-MHC beads (W6.32) for 20 min. Equal amounts of total proteins were immunoprecipitated with 3E1 Mab. The samples were probed by immunoblotting with polyclonal antibodies to the ectodomain of β_4 (top panel) and to Shc (bottom panel). (C) The cytoplasmic domain of β_4 is important for the activation of Shc in response to $\alpha_6\beta_4$ ligation. Clone A and clone L cells were serum starved and either incubated in suspension with medium alone (C), anti- β_4 beads (3E1) or anti-MHC beads (W6.32) for 20 min or plated on dishes coated with anti- β_4 (3E1) or anti-MHC (W6.32) Mab for 30 min. Equal amounts of total proteins were immunoprecipitated with anti-Shc Mab. The samples were probed by immunoblotting with HRP-conjugated anti-P-Tyr Mab RC20 (top panel) or polyclonal anti-Shc antibodies (bottom panel).

of Ras. They also suggest that, in contrast to the sequential recruitment of Shc and Grb2 which can occur in suspended cells treated with anti- β_4 beads, optimal activation of Ras by $\alpha_6\beta_4$ requires adhesion and/or spreading on a substratum coated with $\alpha_6\beta_4$ ligands.

We next examined if ligation of $\alpha_6\beta_4$ resulted in activation of the MAP kinase Erk. Growth factor-starved keratinocytes were detached and either kept in suspension or plated on dishes coated with the anti- β_4 Mab 3E1, the anti-MHC Mab W6.32, laminin 5, collagen I or poly-L-lysine. As a control, adherent keratinocytes were treated with EGF. As shown in Figure 3A, immunoprecipitation

and *in vitro* kinase assays indicated that adhesion to laminin 5- or anti- β_4 Mab-coated dishes causes activation of Erk to a level similar to that induced by EGF. In contrast, adhesion to collagen I, poly-L-lysine or anti-MHC Mab did not result in significant activation of Erk (Figure 3A). Adhesion to anti- α_3 Mab-coated dishes also did not cause Erk activation (not shown). To examine the kinetics of Erk activation by $\alpha_6\beta_4$, keratinocytes were plated on laminin 5 for various times and subjected to Erk immunoprecipitation and kinase assay. As shown in Figure 3B, we detected a significant activation of Erk as early as 5 min after plating the keratinocytes on laminin

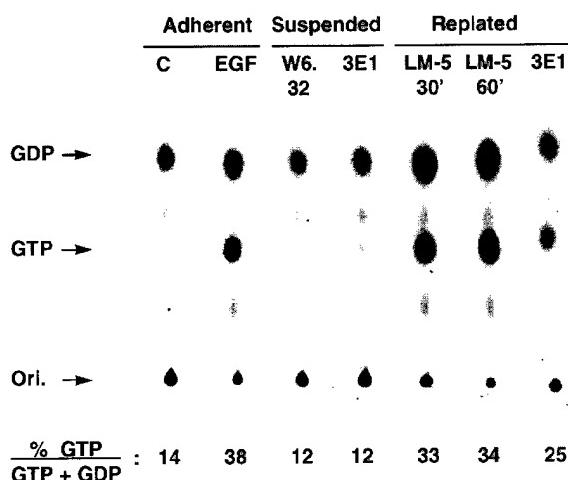


Fig. 2. Activation of Ras by the $\alpha_6\beta_4$ integrin. Primary human keratinocytes were growth factor starved and labeled *in vivo* with [32 P]orthophosphate. After detachment, they were then either incubated in suspension with anti-MHC (W6.32) or anti- β_4 (3E1) Mab-coated beads for 20 min or plated on dishes coated with laminin 5 (LM-5) for the indicated times or on dishes coated with anti- β_4 Mab (3E1) for 30 min. As a control, adherent cells were either left untreated or stimulated with 200 ng/ml EGF for 5 min. After immunoprecipitation of Ras, bound nucleotides were eluted and separated by TLC. Numbers indicate the molar ratio of GTP over total nucleotides.

5. The level of Erk activity peaked at 30 min of adhesion and declined thereafter. These results indicate that ligation of $\alpha_6\beta_4$ causes a significant and relatively persistent activation of the MAP kinase Erk and are in agreement with the observation that ligation of the laminin- and collagen-binding integrins $\alpha_3\beta_1$ and $\alpha_2\beta_1$ does not induce this event (Wary *et al.*, 1996). Since adhesion mediated by $\alpha_3\beta_1$ and $\alpha_2\beta_1$ is known to cause activation of focal adhesion kinase (FAK), these findings are also consistent with the notion that activation of FAK is not sufficient for stimulation of Erk in response to integrin ligation (Wary *et al.*, 1996).

The activation of Erk mediated by $\alpha_6\beta_4$ requires Shc, Ras and Rho

The mechanism of Erk activation in response to $\alpha_6\beta_4$ ligation was examined by testing the effect of various dominant-interfering mutant proteins. Since transient transfection of primary keratinocytes is very inefficient, we elected to use HeLa cells which express levels of $\alpha_6\beta_4$, $\alpha_3\beta_1$ and $\alpha_2\beta_1$ comparable with those of primary keratinocytes. HeLa cells were transfected with a hemagglutinin (HA)-tagged Erk2 vector in combination with different concentrations of cDNAs encoding dominant-negative Shc (317F), Ras (N17), RhoA (N19), CDC42 (N17) and Rac1 (N17). As shown in Figure 4, immunoprecipitation of HA-Erk2 followed by *in vitro* kinase assay indicated that the activation of Erk in response to $\alpha_6\beta_4$ ligation is suppressed by dominant-negative Shc (Figure 4A), Ras and RhoA (Figure 4B), but not by dominant-negative Cdc42 and Rac1 (Figure 4C). Although at the highest concentration tested dominant-negative Shc, Ras and RhoA completely inhibited the activation of Erk by $\alpha_6\beta_4$, at the intermediate and lowest concentration tested the inhibitory activity of the three dominant-negative mutants differed, perhaps as a consequence of the different

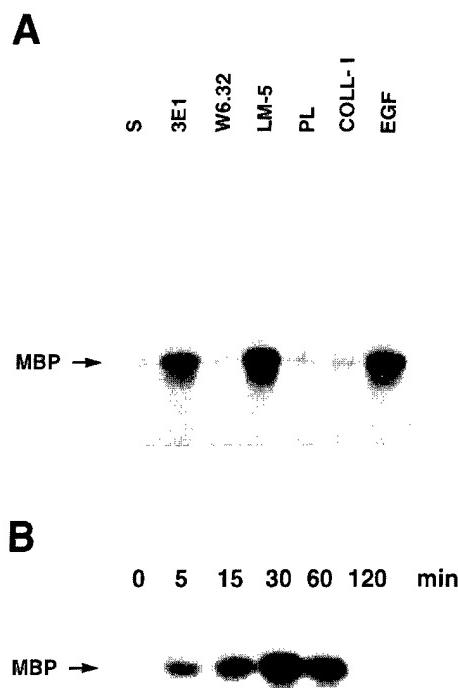


Fig. 3. Activation of Erk kinase by $\alpha_6\beta_4$. (A) Specificity of Erk activation by $\alpha_6\beta_4$. Primary human keratinocytes were growth factor starved, detached and either kept in suspension (S) or plated on dishes coated with anti- β_4 Mab (3E1), anti-MHC Mab (W6.32), laminin 5 (LM-5), collagen I (COLL-I) or poly-L-lysine (PL) for 30 min. As a control, adherent cells were stimulated with 200 ng/ml EGF for 5 min (EGF). (B) Kinetics of Erk activation by $\alpha_6\beta_4$. Primary human keratinocytes were growth factor starved, detached and either kept in suspension or plated on dishes coated with laminin 5 for the indicated times. Anti-Erk immunoprecipitates were subjected to *in vitro* kinase assay using MBP as a substrate.

relative abundance of their target protein or their different mechanism of action. These results indicate that Shc couples the $\alpha_6\beta_4$ integrin to the Ras-Erk signaling pathway and suggest that full activation of Erk in response to $\alpha_6\beta_4$ ligation requires the activity of both Ras and Rho.

Ligation of $\alpha_6\beta_4$ causes activation of the Rac-Jnk signaling pathway

We next examined if ligation of $\alpha_6\beta_4$ stimulated the MAP kinase Jnk. Growth factor-starved primary keratinocytes were detached and either kept in suspension or plated on dishes coated with anti- β_4 Mab, anti-MHC Mab, laminin 5, collagen I or poly-L-lysine. As a control, adherent keratinocytes were exposed to UV light. Jnk was precipitated by using a GST-Jun fusion protein and its activity examined by *in vitro* kinase assay. As shown in Figure 5, the binding of Mab 3E1 or laminin 5 to $\alpha_6\beta_4$ caused an activation of Jnk comparable with that induced by UV stimulation. In contrast, adhesion to dishes coated with collagen I, poly-L-lysine, the control Mab W6.32 (Figure 5) or the anti- $\alpha_3\beta_1$ Mab P1B5 (not shown) did not result in significant activation of Jnk. Time-course experiments indicated that the kinetics of activation of Jnk in response to laminin 5 were similar to those observed for Erk (not shown). These results indicate that ligation of $\alpha_6\beta_4$ causes activation of Jnk, and suggest that ligation of the collagen-binding integrin $\alpha_2\beta_1$ does not induce this event.

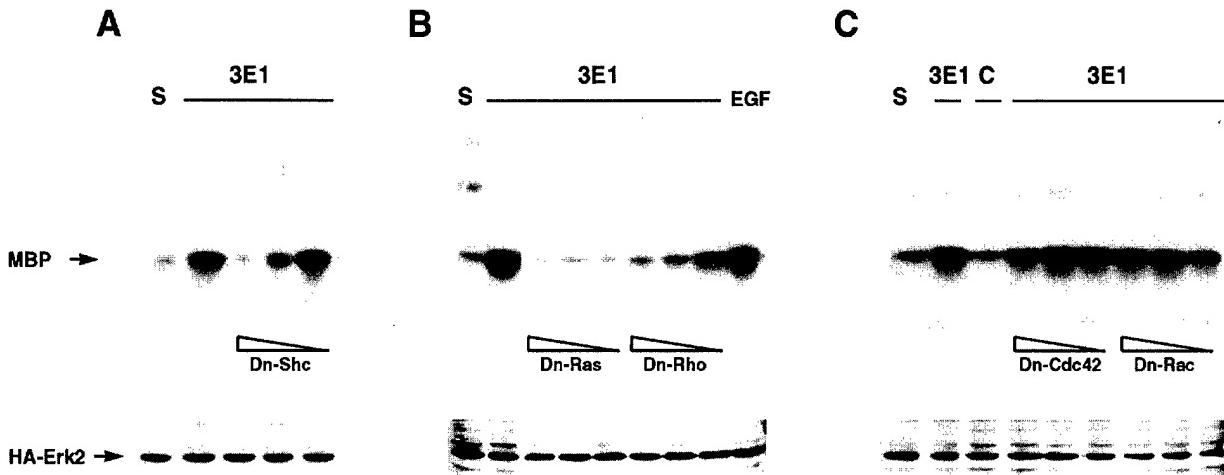


Fig. 4. The activation of Erk caused by $\alpha_6\beta_4$ ligation is inhibited by dominant-negative Shc, Ras and Rho. (A) HeLa cells were transiently transfected with 3 μ g of HA-tagged Erk2 plasmid alone or in combination with 10, 5 and 2.5 μ g of vector encoding dominant-negative Shc (Dn-Shc). The cells were then either kept in suspension (S) or plated on dishes coated with anti- β_4 Mab (3E1) for 30 min. (B) HeLa cells were transiently transfected with 1 μ g of HA-tagged Erk2 plasmid alone or in combination with 1, 0.5 and 0.25 μ g of vectors encoding dominant-negative Ras (Dn-Ras) or RhoA (Dn-Rho). The cells were then either kept in suspension (S) or plated on dishes coated with anti- β_4 Mab (3E1) for 30 min. As a control, adherent cells were treated with 200 ng/ml EGF for 5 min. (C) HeLa cells were transiently transfected with 1 μ g of HA-tagged Erk2 plasmid alone or in combination with 1, 0.5 and 0.25 μ g of vectors encoding dominant-negative Cdc42 (Dn-Cdc42) or Rac (Dn-Rac). The cells were either kept in suspension (S) or plated on dishes coated with anti- β_4 Mab (3E1) or anti-MHC Mab (C) for 30 min. Anti-HA immunoprecipitates were subjected to *in vitro* kinase assay using myelin basic protein (MBP) as a substrate (top panels). Transfection efficiencies were verified by immunoblotting aliquots of total proteins with anti-HA antibodies (bottom panels).

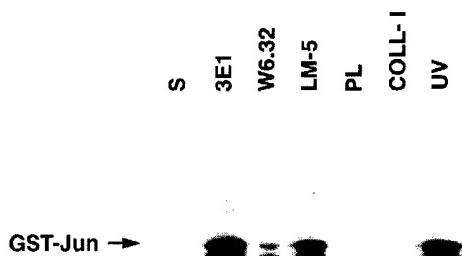


Fig. 5. Activation of Jnk kinase by $\alpha_6\beta_4$. Primary human keratinocytes were growth factor starved, detached and either kept in suspension (S) or plated on dishes coated with anti- β_4 Mab (3E1), anti-MHC Mab (W6.32), laminin 5 (LM-5), poly-L-lysine (PL) or collagen I (COLL-I) for 20 min. As a control, adherent cells were exposed to 40 J/m^2 of UV radiation (UV) and then kept in culture for 20 min. Jnk kinase was precipitated using glutathione beads coated with GST-Jun fusion protein and subjected to *in vitro* kinase assay. The position of phosphorylated GST-Jun is indicated. The lower band is a degradation product of GST-Jun.

The activation of Jnk mediated by $\alpha_6\beta_4$ requires Ras, PI-3K and Rac

The mechanism of Jnk activation in response to $\alpha_6\beta_4$ ligation was examined by testing the effect of various dominant-interfering mutant proteins and the phosphoinositide 3-kinase (PI-3K) inhibitor Wortmannin. HeLa cells were transfected with a Flag-tagged Jnk1 vector in combination with different concentrations of cDNAs encoding dominant-negative Ras (N17), Rac1 (N17), Cdc42 (N17) and RhoA (N19). As shown in Figure 6A, precipitation of Flag-Jnk1 followed by *in vitro* kinase

assay indicated that the activation of Jnk in response to $\alpha_6\beta_4$ ligation is suppressed by dominant-negative Ras and Rac1, but not by dominant-negative RhoA, and very modestly by dominant-negative Cdc42. These results indicate that the activation of Jnk by $\alpha_6\beta_4$ requires the activity of both Ras and Rac. Although it has been suggested that Raf can activate Jnk by acting on the MAP kinase kinase kinase MEKK1 (Lange-Carter and Johnson, 1994), recent results indicate that the predominant mechanism by which Ras activates Jnk involves Rac, and not Raf (Kosravi-Far *et al.*, 1995; Minden *et al.*, 1995; Qiu *et al.*, 1995; Joneson *et al.*, 1996). The requirement for Rac in our system is consistent with this hypothesis. Since PI-3K is a downstream target effector of Ras (Rodriguez-Viciana *et al.*, 1994) and has been implicated in the activation of Rac (Nobes *et al.*, 1995; Klippel *et al.*, 1996), we tested if inhibition of PI-3K interfered with the activation of Jnk by $\alpha_6\beta_4$. As shown in Figure 6B, the activation of Jnk in response to ligation of $\alpha_6\beta_4$ was completely suppressed by as little as 50 nM Wortmannin, a concentration at which the inhibitor has very little effect on signaling molecules other than PI-3-K (Wymann *et al.*, 1996). Taken together, the results of these experiments suggest that $\alpha_6\beta_4$ activates the Rac-Jnk signaling pathway via Ras and PI-3K.

Induction of Fos SRE-dependent transcription by $\alpha_6\beta_4$

We next examined if the coupling of $\alpha_6\beta_4$ to Shc played a role in the control of immediate-early gene expression. Since Erk regulates transcription from the Fos serum response element (SRE) by phosphorylating the ternary complex factors Elk-1 and SAP-1, and Rho family proteins cooperate with this Erk function by acting on the serum response factor (Treisman, 1995), we examined the effect of $\alpha_6\beta_4$ ligation on the Fos SRE. HeLa cells were

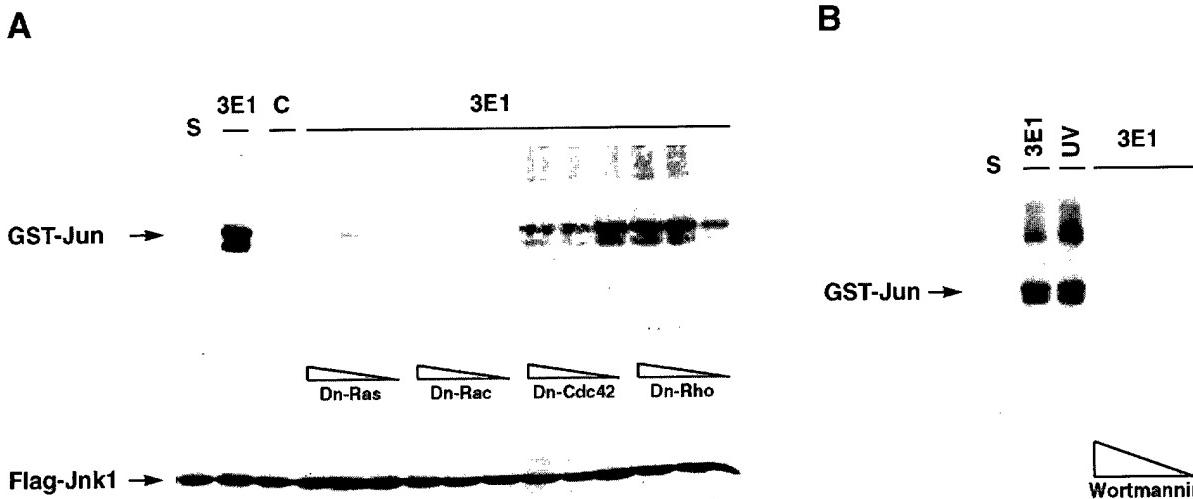


Fig. 6. The activation of Jnk kinase caused by $\alpha_6\beta_4$ ligation is inhibited by dominant-negative versions of Ras and Rac and by the PI-3K inhibitor Wortmannin. (A) HeLa cells were transiently transfected with 1 μ g of Flag-tagged Jnk1 plasmid alone or in combination with 1, 0.5 and 0.25 μ g of vectors encoding dominant-negative Ras (Dn-Ras), Rac (Dn-Rac), Cdc42 (Dn-Cdc42) and RhoA (Dn-Rho). The cells were then either kept in suspension or plated on dishes coated with anti- β_4 Mab (3E1) or anti-MHC Mab (C) for 20 min. Flag-Jnk was immunoprecipitated with the anti-Flag Mab M2 and subjected to *in vitro* kinase assay with GST-Jun as a substrate (top panel). Transfection efficiencies were verified by immunoblotting aliquots of total proteins with Mab M2 (bottom panel). (B) HeLa cells were transiently transfected with 1 μ g of Flag-tagged Jnk1 plasmid. After detachment, the cells were either kept in suspension or plated on dishes coated with anti- β_4 Mab (3E1) in the absence or presence of 200, 100 or 50 nM Wortmannin for 20 min. As a control, adherent cells were exposed to 40 J/m² of UV radiation for 20 min. Flag-Jnk was immunoprecipitated with Mab M2 and subjected to *in vitro* kinase assay with GST-Jun as a substrate. The position of phosphorylated GST-Jun is indicated. The lower band is a degradation product of GST-Jun.

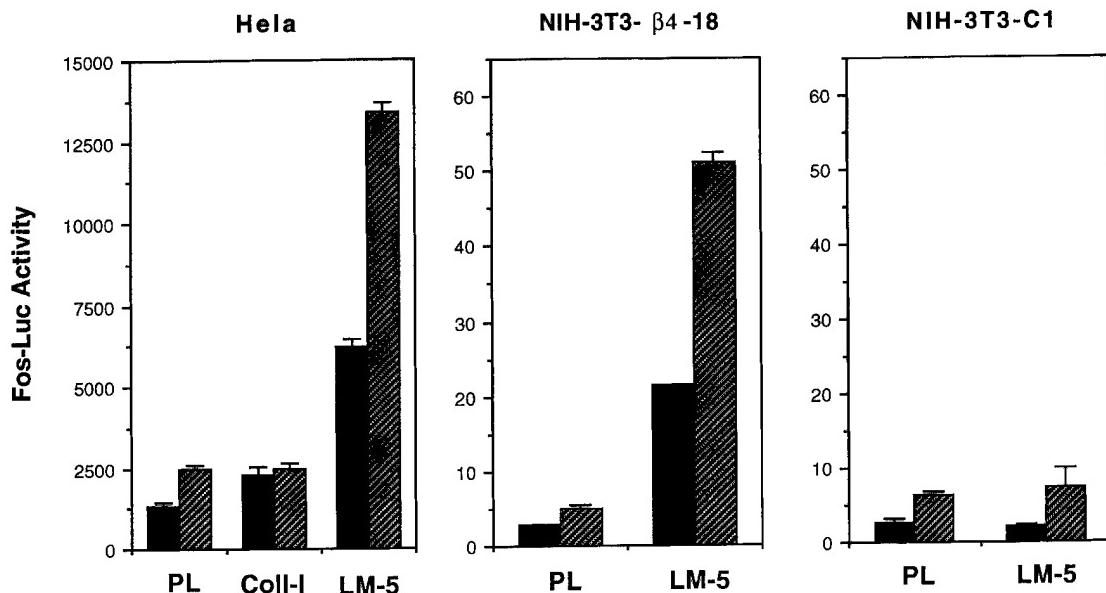


Fig. 7. Adhesion mediated by $\alpha_6\beta_4$ promotes transcription from the Fos SRE. HeLa cells, β_4 -expressing NIH 3T3- β_4 -18 cells and control NIH 3T3-C1 cells were transiently transfected with Fos-SRE-Luc plasmid. After growth factor starvation, the cells were detached and plated onto dishes coated with 10 μ g/ml poly-L-lysine (PL), laminin 5 (LM-5) or collagen I (Coll-I) for 30 min. The cells were then either left untreated (solid bars) or exposed to mitogens for 10 min (shaded bars). Cell lysates were subjected to luciferase assay. Values are expressed in arbitrary units. The diagram shows the mean value and standard deviation from triplicate samples.

transiently transfected with the Fos-SRE-Luc vector, which contains the Fos SRE promoter element linked to the luciferase reporter gene. Upon plating on dishes coated with poly-L-lysine, collagen I or laminin 5, the cells were either left untreated or exposed to EGF. They were then subjected to luciferase assay. As shown in Figure 7, while adhesion to laminin 5 in the absence of EGF caused elevation of Fos SRE-dependent transcription, adhesion to poly-L-lysine or collagen I under the same conditions

did not induce this activity. This suggests that ligation of $\alpha_6\beta_4$, but not $\alpha_6\beta_1$, is sufficient to promote transcription from the Fos SRE in the absence of mitogens. Treatment with EGF induced a significant elevation of Fos SRE activity in HeLa cells adhering to laminin 5, but caused a remarkably modest effect in cells attaching to poly-L-lysine or collagen I. This result suggests that ligation of $\alpha_6\beta_4$ is required for optimal induction of Fos SRE-dependent transcription in response to EGF.

To examine if laminin 5 is able to induce transcription from the Fos SRE in normal untransformed cells and to demonstrate the role of $\alpha_6\beta_4$ in this process, we transfected NIH 3T3 fibroblasts with a retroviral vector encoding human β_4 and isolated stable cell lines. Immunoprecipitation and fluorescence-activated cell sorting (FACS) analysis indicated that the recombinant β_4 subunit associated with endogenous α_6 and was regularly exported to the cell surface. Clones 8 and 18 displayed the highest levels of recombinant β_4 on the cell surface and were examined further. FACS analysis indicated that the level of expression of recombinant β_4 in these two clones approximated 40% of that of endogenous β_4 in primary keratinocytes. Since the NIH 3T3 cells do not express $\alpha_6\beta_4$ and adhere to laminin 5 through $\alpha_3\beta_1$, we examined the function of recombinant $\alpha_6\beta_4$ by comparing the kinetics by which the β_4 transfectants and control cells adhered to laminin 5-coated dishes. The results showed that the β_4 transfectants adhered to laminin 5 with faster kinetics than the controls. However, both types of cells became equally spread by 30 min of plating (not shown).

To analyze Fos SRE-dependent transcription in response to laminin 5, control and β_4 -transfected NIH 3T3 cells were transiently transfected with the Fos-SRE-Luc plasmid. Upon plating on dishes coated with poly-L-lysine or laminin 5, the cells were either left untreated or exposed to basic fibroblast growth factor (bFGF), insulin and platelet-derived growth factor (PDGF). The results of luciferase assays indicated that adhesion to laminin 5 causes elevation of Fos SRE-dependent transcription in the β_4 transfectants even in the absence of mitogens and this induction is potentiated by mitogen treatment (Figure 7). The ability of laminin 5 to induce Fos SRE-dependent transcription was dependent on $\alpha_6\beta_4$ expression, because adhesion to laminin 5 did not cause this effect in control cells even after mitogen treatment (Figure 7). These results indicate that ligation of $\alpha_6\beta_4$, but not $\alpha_3\beta_1$, is sufficient to induce Fos SRE-dependent transcription, and confirm that $\alpha_6\beta_4$ cooperates with mitogens to cause optimal induction of this activity.

Adhesion mediated by $\alpha_6\beta_4$ promotes cell cycle progression

To examine if $\alpha_6\beta_4$ signaling played a role in cell cycle progression, primary keratinocytes were growth factor starved and then plated in the presence of EGF on plastic wells coated with laminin 5, collagen I or poly-L-lysine. Entry into the S phase was examined by 5'-bromo-2'-deoxyuridine (BrdU) incorporation and anti-BrdU staining. As shown in Figure 8, a significant fraction of keratinocytes plated on laminin 5 entered into the S phase during the 22 h of the assay. In contrast, only a modest percentage of cells plated on collagen I or poly-L-lysine entered into S during the assay. In the absence of mitogens, a similarly small percentage of cells plated on collagen I or poly-L-lysine entered into S phase (not shown). This fraction may consist of unsynchronized cells, which have already passed the G₁-S boundary at the time of plating. In addition, because the keratinocytes acquired and maintained a well-spread morphology on collagen I, their inability to enter into S on this substratum is not the result of insufficient spreading. The results of these experiments indicate that physical attachment and spreading on the

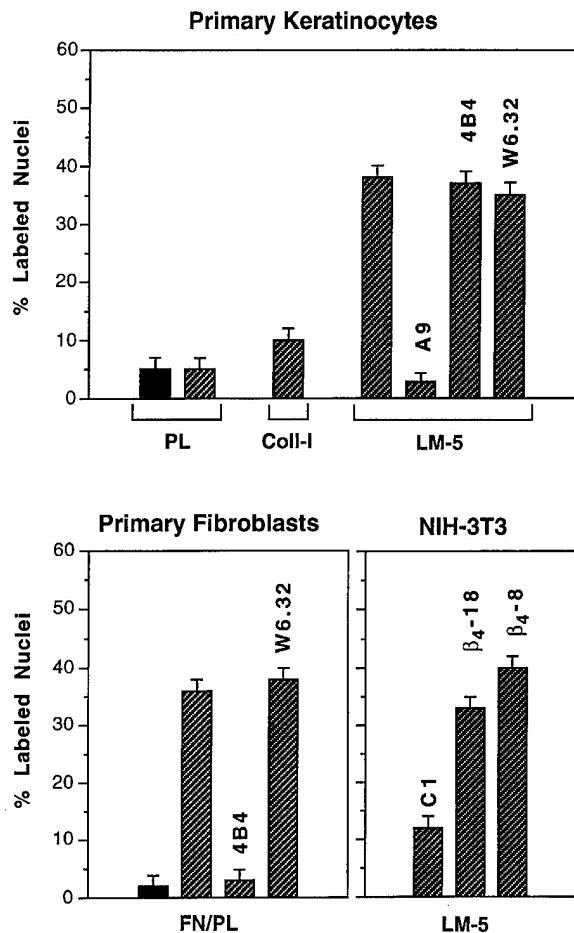


Fig. 8. Adhesion mediated by $\alpha_6\beta_4$ promotes cell cycle progression. Primary human keratinocytes, primary human dermal fibroblasts, control NIH 3T3-C1 cells and β_4 -expressing NIH 3T3-18 and -8 cells were growth factor starved and plated on wells coated with 10 μ g/ml poly-L-lysine (PL), collagen I (Coll-I) or laminin 5 (LM-5), or a mixture of 10 μ g/ml fibronectin and 10 μ g/ml poly-L-lysine (FN/PL). The cells were then incubated for 22 h in defined medium containing 10 μ M BrdU and mitogens (shaded bars). To estimate the percentage of unsynchronized primary cells, the cells were also incubated for 22 h in defined medium containing 10 μ M BrdU without mitogens (solid bars). When indicated, 10 μ g/ml anti- β_1 Mab 4B4, 1:20 anti- β_4 Mab A9 ascites or 10 μ g/ml control anti-MHC Mab W6.32 were included in the medium. After immunostaining with anti-BrdU Mab and alkaline phosphatase-conjugated secondary antibodies, the percentage of labeled nuclei was determined by scoring at least 500 cells from five different microscopic fields. The diagram shows the mean value and standard deviation from triplicate samples.

extracellular matrix is not sufficient for progression of keratinocytes through G₁ in response to EGF, and suggest that this process requires ligation of a specific integrin, such as $\alpha_6\beta_4$.

To examine the relative roles of $\alpha_6\beta_4$ and $\alpha_3\beta_1$ in keratinocyte proliferation, we tested the effect of inhibitory anti- β_4 and anti- β_1 antibodies. Growth factor-starved keratinocytes were plated on laminin 5 and exposed to EGF in the presence of the inhibitory anti- β_1 Mab 4B4, the inhibitory anti- β_4 Mab A9 or the control anti-MHC Mab W6.32. As shown in Figure 8, exposure to the anti- β_4 Mab completely suppressed keratinocyte entry into S. In contrast, treatment with the anti- β_1 or anti-MHC Mab did not inhibit keratinocyte proliferation on laminin 5. To control the efficacy of the anti- β_1 Mab 4B4, G₀ synchronized

primary human fibroblasts were plated on a mixed substrate consisting of poly-L-lysine and fibronectin and exposed to mitogens in the presence of the 4B4 or W6.32 Mab. In accordance with the recent observation that a class of β_1 integrins, which include the $\alpha_5\beta_1$ fibronectin receptor, is linked to the Ras-Erk pathway and the control of cell cycle progression by Shc (Wary *et al.*, 1996), plating of the primary fibroblasts on fibronectin/poly-L-lysine promoted cell cycle progression, and exposure to anti- β_1 Mab 4B4 blocked this process without inducing detachment (Figure 8). These results suggest that the ability of laminin 5 to promote keratinocyte cell cycle progression is mediated by $\alpha_6\beta_4$, and not by $\alpha_3\beta_1$.

We next examined the ability of control and β_4 -expressing NIH 3T3 cells to progress through G₁ on laminin 5. While only a modest percentage of control cells progressed through G₁ when plated on laminin 5 for 22 h, a significant fraction of β_4 expressors entered into S under the same conditions (Figure 8), suggesting that ligation of $\alpha_6\beta_4$ is sufficient to promote progression through G₁ in response to mitogens. Taken together, the results of these assays indicate that ligation of $\alpha_6\beta_4$ is required and sufficient to promote keratinocyte proliferation in response to laminin 5.

Discussion

Although the notion that cell adhesion to the extracellular matrix regulates gene expression is supported by considerable experimental evidence, the signaling pathways linking integrins to nuclear events are not well known. In particular, the mechanisms by which integrin-dependent signals regulate cell cycle progression in normal epithelial cells are not fully understood. The results of recent studies have defined the membrane-proximal events induced by ligation of the $\alpha_6\beta_4$ integrin, a laminin receptor involved in various morphogenetic processes (Giancotti, 1996). Upon binding to extracellular ligand, $\alpha_6\beta_4$ becomes phosphorylated on tyrosine residues by the action of an integrin-associated kinase and thereby combines sequentially with the adaptor proteins Shc and Grb2 (Mainiero *et al.*, 1995). The results of the present study provide clear evidence that these receptor-proximal events result in the activation of Ras and of two distinct MAP kinase signaling pathways which regulate immediate-early gene expression. In contrast, other integrins, such as the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ collagen and laminin receptors, do not induce these events. Since $\alpha_6\beta_4$ -mediated adhesion promotes keratinocyte progression through G₁ in response to growth factor treatment, while $\alpha_2\beta_1$ - and $\alpha_3\beta_1$ -mediated adhesion does not, we propose that the linkage of $\alpha_6\beta_4$ to Ras signaling mediated by Shc participates in the control of cell cycle progression in normal epithelial cells.

The adaptor protein Shc contains two separate domains involved in the recognition of tyrosine-phosphorylated sequence motifs: an N-terminal phosphotyrosine-binding (PTB) domain and a C-terminal Src homology 2 (SH2) domain (Pawson, 1995). GST fusion protein binding experiments have suggested that both domains can interact with the tyrosine-phosphorylated β_4 tail (Mainiero *et al.*, 1995). The results of this study are in accordance with the notion that the recruitment of Shc to $\alpha_6\beta_4$ is mediated by the cytoplasmic domain of β_4 and suggest that this event

is important for the subsequent tyrosine phosphorylation of Shc, which is presumably mediated by the integrin-associated kinase. Upon phosphorylation, Shc combines with the other adaptor protein Grb2. Since Grb2 is constitutively associated with the Ras-GTP exchange factor mSOS, the recruitment of Grb2 to the plasma membrane potentially links $\alpha_6\beta_4$ to Ras. In accordance with this hypothesis, our current results show that ligation of $\alpha_6\beta_4$ results in a significant activation of Ras.

In contrast to the recruitment of Shc and Grb2 which could be observed in suspended keratinocytes cross-linked with anti- $\alpha_6\beta_4$ antibodies, full activation of Ras required physical attachment and/or spreading on a substratum coated with $\alpha_6\beta_4$ ligands. Since it has been suggested that proper targeting of the Grb2-mSOS complex to Ras may require an interaction of the Grb2 SH3 domains with the cortical cytoskeleton (Bar-Sagi *et al.*, 1993), it is possible that such targeting is defective in suspended keratinocytes cross-linked with anti- $\alpha_6\beta_4$ antibodies. Alternatively, since the pleckstrin homology domain of mSOS may bind to phosphatidylinositol(4,5)bisphosphate (PtdInsP₂) in the plasma membrane (Lemmon *et al.*, 1996) and it is known that PtdInsP₂ levels decline in suspended cells (McNamee *et al.*, 1993), it is possible that Ras activation by $\alpha_6\beta_4$ requires a threshold concentration of PtdInsP₂ in the plasma membrane which is not available in suspended keratinocytes. Future studies will be required to resolve this issue.

The results of this study indicate that ligation of $\alpha_6\beta_4$ results in the stimulation of both Ras-Erk and Rac-Jnk MAP kinase signaling pathways. The activation of Erk by $\alpha_6\beta_4$ was suppressed by dominant-negative versions of both Shc and Ras, indicating that the coupling to Ras mediated by Shc is the major mechanism by which $\alpha_6\beta_4$ controls Erk activation. Interestingly, Erk activation was also inhibited by dominant-negative RhoA. This result, which is in agreement with the recent observation that Rho activity is required for full activation of Erk in response to various extracellular stimuli (Hill *et al.*, 1995), suggests that this G protein also participates in signaling by $\alpha_6\beta_4$. The activation of Jnk by $\alpha_6\beta_4$ was inhibited by dominant-negative Ras and Rac1, but not by dominant-negative RhoA and Cdc42. In addition, it was suppressed by nanomolar concentrations of the PI-3K inhibitor Wortmannin. Since there is evidence that PI-3K is a downstream target effector of Ras and is involved in the activation of Rac (Rodriguez-Viciana *et al.*, 1994; Nobes *et al.*, 1995; Klipper *et al.*, 1996), it is likely that $\alpha_6\beta_4$ stimulates the Rac-Jnk pathway via Ras. Thus, the coupling of $\alpha_6\beta_4$ to Ras mediated by Shc leads to the activation of both Ras-Erk and Rac-Jnk signaling pathways.

In accordance with the observation that Erk stimulates transcription of the immediate-early gene *fos* (Treisman, 1995), the results of our study indicate that adhesion mediated by $\alpha_6\beta_4$ is sufficient to promote transcription from the Fos SRE. Interestingly, while treatment with mitogens caused a significant elevation of Fos SRE activity in cells plated on the $\alpha_6\beta_4$ ligand laminin 5, it was ineffective in cells adhering to the $\alpha_2\beta_1$ ligand collagen I, indicating that the expression of Fos in response to mitogens requires ligation of a specific integrin, such as $\alpha_6\beta_4$. Future studies will be required to examine further the mechanism by which $\alpha_6\beta_4$ controls immediate-early

gene expression. For example, it is known that Rho family proteins can activate the Fos promoter by stimulating the serum response factor (Hill *et al.*, 1995). The ability of $\alpha_6\beta_4$ to stimulate Rac may thus contribute to the activation of Fos promoter in response to laminin 5. In addition, since it is well established that Jnk controls the activity of the Jun promoter (Karin, 1995), it is likely that $\alpha_6\beta_4$ also regulates the expression of the immediate-early expression gene Jun. Taken together, these observations suggest that $\alpha_6\beta_4$ is a crucial regulator of immediate-early gene expression.

What is the biological significance of $\alpha_6\beta_4$ signaling? The results of our cell proliferation analysis indicate that $\alpha_6\beta_4$ signaling promotes transit through G₁ in keratinocytes and other $\alpha_6\beta_4$ -expressing cells exposed to mitogens. In this respect, $\alpha_6\beta_4$ appears to be functionally distinct from other integrins, such as $\alpha_3\beta_1$ and $\alpha_2\beta_1$, which do not appear to be able to do so. In fact, it is quite remarkable that keratinocytes plated on the $\alpha_2\beta_1$ ligand collagen I adhere and spread but do not enter into the S phase despite being exposed to otherwise mitogenic concentrations of EGF. We have observed recently that a class of β_1 and α_v integrins, which include $\alpha_1\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$, but not $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_1$, are also linked to the Ras-Erk pathway and the control of cell cycle progression by Shc (Wary *et al.*, 1996). Taken together, these observations suggest that the ability of extracellular matrix to control cell proliferation, thereby mediating anchorage-dependent cell growth, depends on its composition and the repertoire of integrins on the responding cell.

What is the mechanism by which $\alpha_6\beta_4$ signaling regulates cell proliferation? Previous studies have indicated that the cell cycle of normal cells contains an anchorage-dependent transition in early-mid G₁. In fact, adhesion to the extracellular matrix is required for translation of cyclin D₁ in cells exposed to mitogens, suggesting that integrin- and growth factor-dependent signals converge prior to the induction of cyclin D₁ to control progression through G₁ (Fang *et al.*, 1996; Zhu *et al.*, 1996). Our results suggest that these signals are integrated before the induction of immediate-early gene expression. The simplest hypothesis is that in normal cells growth factor receptors and specific integrins cooperate to activate MAP kinase beyond the threshold level required for immediate-early gene expression. Since most dominant oncogenes, including Shc (Pelicci *et al.*, 1992), induce neoplastic transformation by constitutively activating the Ras-MAP kinase pathway, this model also explains why neoplastic cells usually display anchorage-independent growth.

In conclusion, the results of this study indicate that the coupling of $\alpha_6\beta_4$ integrin to Ras-Erk and Rac-Jnk pathways mediated by Shc regulates immediate-early gene expression and cell cycle progression in response to mitogens. Since the major keratinocyte integrins, in addition to $\alpha_6\beta_4$, are $\alpha_2\beta_1$ and $\alpha_3\beta_1$, which are not coupled to Shc, the signaling function of $\alpha_6\beta_4$ may explain why cell proliferation is restricted to the basal layer in the epidermis and other stratified epithelia. In addition, since exit from the cell cycle is a prerequisite for differentiation, our results may also explain why the onset of keratinocyte differentiation is coupled to the detachment from the basement membrane (Hall and Watt, 1989). Finally, the ability of $\alpha_6\beta_4$ to stimulate cell growth suggests that

its overexpression in squamous carcinoma (Kimmel and Carey, 1986; Wolf *et al.*, 1990) may contribute to tumor progression.

Materials and methods

Antibodies and extracellular matrix molecules

The specificity of anti- β_4 Mab 3E1 was described previously (Giancotti *et al.*, 1992). The polyclonal antiserum to the β_4 ectodomain was generated by immunizing a rabbit with a GST fusion protein comprising amino acids 31–217. The inhibitory anti- β_4 Mab A9 was obtained from Tom Carey (Comprehensive Cancer Center, University of Michigan at Ann Arbor). Hybridomas producing the anti- α_1 Mab TS2/7 and anti- β_1 Mab TS2/16 were obtained from ATCC (Rockville, MD). The anti- α_2 Mab P1E6, anti- α_3 Mab P1B5 and anti- α_5 Mab P1D6 were from Gibco-BRL (Gaithersburg, MD). The anti- β_1 Mab 4B4 was from Coulter (Hialeah, FL). The anti-MHC Mab W6.32 reacts with human and cultured rodent cells. The anti-FLAG M2 Mab and anti-HA peptide tag Mab 12CA5 were purchased from Eastman Kodak Company (New Haven, CT) and Boehringer Mannheim (Indianapolis, IN), respectively. The anti-Shc Mab and the recombinant horseradish peroxidase (HRP)-conjugated anti-P-Tyr Mab RC20 were from Transduction Laboratories (Lexington, KY). Anti-Erk2 and anti-Grb2 polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Human fibronectin and collagen I were purchased from Gibco-BRL. Laminin 5 matrices were prepared as described previously (Sonnenberg *et al.*, 1993; Spinardi *et al.*, 1995).

Cell lines, constructs and transfections

HeLa cells were cultured in Dulbecco's modified minimal essential medium (DMEM) with 10% fetal calf serum (FCS). Primary human keratinocytes were cultured in keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract (50 µg/ml) and human recombinant EGF (5 ng/ml) (Gibco BRL). NIH 3T3 cells expressing recombinant human β_4 were maintained in DMEM containing 10% bovine calf serum (BCS) and 3 mM L-histidinol (Sigma, St Louis, MO). Primary dermal fibroblasts were obtained from Clonetics (San Diego, CA) and cultured in fibroblast basal medium (FBM) supplemented with 2% FCS, 2 ng/ml bFGF and 10 µg/ml insulin.

To generate pLXSHD- β_4 , the full-length human β_4 cDNA was subcloned in the EcoRI site of pLXSHD, a derivative of the Moloney leukemia virus-derived retroviral vector pLXSN containing as a selection marker the histidinol dehydrogenase gene (Dusty Miller and Rosman, 1989). The recombinant virus was produced by transiently transfected 293-T cells with 10 µg of pLXHD- β_4 and 10 µg of packaging-defective ecotropic virus (Landau and Littman, 1992). Subconfluent NIH 3T3 cells were infected with a dilution of the culture supernatant collected 48 h after transfection. Cell lines expressing recombinant human β_4 were isolated by L-histidinol selection and identified by FACS analysis. Rat 804G cells expressing a recombinant wild-type human β_4 subunit were previously described (Spinardi *et al.*, 1993). To generate a cytomegalovirus (CMV)-driven eukaryotic expression vector encoding a tail-less β_4 subunit, the 2.3 kb EcoRI-Scal fragment of β_4 cDNA was ligated into EcoRI-BamHI-digested pRK-5. The 2.3 kb EcoRI-XbaI fragment of the resulting plasmid was then ligated into EcoRI-XbaI-linearized pRc-CMV. The recombinant β_4 subunit encoded by this vector is truncated immediately after Lys734, which marks the boundary between the transmembrane and intracellular domains of the polypeptide. Clone L cells were generated by transfecting parental 804G cells with the above vector according to previously published protocols (Spinardi *et al.*, 1993). FACS analysis was used to verify that clone A and clone L cells had comparable levels of expression of recombinant β_4 . Metabolic labeling with [³⁵S]methionine/cysteine (Translabel, ICN, Costa Mesa, CA) and immunoprecipitation were used to verify the correct assembly of recombinant β_4 with endogenous α_6 in both NIH 3T3 and 804G transfectants.

The CMV promoter-based expression vectors encoding HA-tagged Erk2, dominant-negative p52^{Shc} (Y317F) and dominant-negative Ras (N17) were obtained from Edward Scolnick (NYU School of Medicine). The vectors encoding GST-Jun and Flag-tagged Jnk1 were described previously (Hibi *et al.*, 1993; Derijard *et al.*, 1994). The cDNAs encoding dominant-negative RhoA (N19) and Rac1 (N17) (Khosravi-Far *et al.*, 1995) were subcloned in pcDNA3. Dominant-negative Cdc42 (N17) in pCMV5 was obtained from Jonathan Chernoff (Fox Chase Cancer Center, Philadelphia, PA). The Fos-SRE-Luc reporter plasmid was from

Joseph Schlessinger. Vectors were transiently transfected in HeLa and NIH 3T3 cells by the lipofectamine method (Gibco-BRL).

Biochemical methods

To obtain ligation of integrins in the absence of any co-stimulus, the cells were growth factor starved for 36 h, detached, and resuspended in serum-free medium. The cells were then either incubated in suspension with polystyrene beads (2.5 μ m diameter, IDC, Portland, OR) coated with anti-integrin Mabs (Mainiero et al., 1995; Wary et al., 1996) or plated onto dishes coated sequentially with affinity-purified goat anti-mouse IgGs and anti-integrin Mabs or extracellular matrix proteins. At the coating concentrations used, the cells attached and spread equally well on laminin 5 and collagen I, attached and partially spread on the anti- β_4 Mab 3E1, and attached without spreading on poly-L-lysine and the control Mab W6.32. As a positive control for Jnk activation, adherent cells were exposed to UV radiation as previously described (Hibi et al., 1993; Derijard et al., 1994). At the end of the incubation, the cells were extracted and subjected to biochemical analysis.

To immunoprecipitate Shc and $\alpha_6\beta_4$, primary human keratinocytes were extracted in Triton lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100) containing 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 25 mM sodium fluoride, 0.01% aprotinin, 4 mg/ml pepstatin A, 10 mg/ml leupeptin and 1 mM phenylmethanesulfonyl fluoride (PMSF) (all from Sigma) for 30 min on ice. Immunoprecipitation, SDS-PAGE and immunoblotting analysis were performed as previously described (Giancotti and Ruoslahti, 1990; Mainiero et al., 1995). Nitrocellulose-bound antibodies were detected by chemiluminescence with ECL (Amersham Life Sciences, Little Chalfont, UK).

To examine Erk activity, cells were extracted with NP-40 lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA) containing phosphatase and protease inhibitors for 30 min on ice. Endogenous and recombinant tagged Erks were immunoprecipitated with anti-Erk2 or anti-HA Mab, respectively, and subjected to *in vitro* kinase assay. The kinase reaction was initiated by adding to the beads 25 μ l of kinase buffer (25 mM Tris pH 7.5, 12.5 mM β -glycerophosphate, 7.5 mM MgCl₂, 20 μ M cold ATP, 0.5 mM sodium orthovanadate) containing 5 μ Ci of [γ -³²P]ATP (4500 Ci/mmol, ICN Biomedicals Inc.) and 2.5 μ g of myelin basic protein (Sigma). After 30 min of incubation at 30°C, the samples were boiled in sample buffer and separated by SDS-PAGE.

To analyze the activation of Jnk, cells were extracted for 30 min on ice with modified Triton lysis buffer (25 mM HEPES pH 7.5, 300 mM NaCl, 0.1% Triton X-100, 0.2 mM EDTA, 20 mM β -glycerophosphate, 1.5 mM MgCl₂, 0.5 mM dithiothreitol) containing phosphatase and protease inhibitors. Endogenous Jnk was precipitated with 3 μ g of GST-Jun fusion protein coupled to glutathione-agarose beads. After washing, the beads were incubated with 25 μ l of kinase buffer containing 5 μ Ci of [γ -³²P]ATP. Recombinant Flag-tagged Jnk1 was immunoprecipitated with the anti-Flag Mab M2 and incubated with 25 μ l of kinase buffer containing 2.5 μ g of GST-Jun and 5 μ Ci of [γ -³²P]ATP. After 30 min of incubation at 30°C, the samples were boiled in sample buffer and separated by SDS-PAGE.

To estimate Ras activation, primary human keratinocytes were starved for 48 h in K-SFM and labeled for 12 h with [³²P]orthophosphate (0.5 mCi/ml, ICN) in phosphate-free DMEM supplemented with 0.1% phosphate-free BCS. After stimulation, the cells were extracted and the samples subjected to Ras-GTP loading assay as described previously (Gale et al., 1993). Nucleotides bound to Ras were analyzed by TLC on PEI-cellulose plates in 0.75 M K₂HPO₄, pH 3.5. Radioactivity in GDP and GTP was estimated by Phosphorimager analysis.

To measure transcription from the Fos SRE, HeLa cells and NIH 3T3 transfectants were transiently transfected with the reporter plasmid Fos-SRE-Luc. After 24 h of growth factor starvation, the cells were detached and plated on dishes coated with laminin 5, collagen I or poly-L-lysine for 30 min. The HeLa cells were then either left untreated or exposed to 50 ng/ml EGF, 20 ng/ml PDGF-BB, 2 ng/ml bFGF and 10 μ g/ml insulin for 10 min. The NIH 3T3 transfectants were either left untreated or exposed to 10 ng/ml PDGF-BB, 5 ng/ml bFGF and 10 μ g/ml insulin for 10 min. Luciferase activity in cell lysates was estimated as previously described (Brasier et al., 1989).

Measurement of cell cycle progression

To monitor progression through G₁ and entry into S phase, the cells were starved by incubation in medium devoid of serum and growth factors for 48 h, detached and plated at low density on microtiter plates or glass coverslips coated with 10 μ g/ml poly-L-lysine, laminin 5, collagen I or a mixture of 10 μ g/ml fibronectin and 10 μ g/ml poly-L-

lysine. The keratinocytes were incubated in K-SFM supplemented with 5 ng/ml human recombinant EGF. The NIH 3T3 transfectants were incubated in FBM supplemented with 20 ng/ml PDGF, 2 ng/ml bFGF and 10 μ g/ml insulin. Primary dermal fibroblasts were incubated in FBM with 2 ng/ml bFGF and 10 μ g/ml insulin. The media were supplemented with 10 μ M BrdU and, when indicated, with the inhibitory anti- β_1 Mab 4B4, the inhibitory anti- β_4 Mab A9 or the control anti-MHC Mab W6.32. After 22 h of incubation, the cells were fixed in 70% ethanol, 50 mM glycine, pH 2.0 for 30 min at -20°C and stained with anti-BrdU Mab and alkaline phosphatase-conjugated secondary antibodies (Boehringer Mannheim, Indianapolis, IN). The percentage of labeled nuclei was determined by scoring at least 500 cells from five different microscopic fields.

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Integrin signaling: specificity and control of cell survival and cell cycle progression

Filippo G Giancotti

Integrin-mediated adhesion to the extracellular matrix plays an important role in regulating cell survival and proliferation. There is now increasing evidence that integrins activate shared as well as subgroup-specific signaling pathways. The signals from these adhesion receptors are integrated with those originating from growth factor and cytokine receptors in order to organize the cytoskeleton, stimulate mitogen-activated protein kinase cascades, and regulate immediate early gene expression. The repertoire of integrins and composition of the extracellular matrix appear to dictate whether a cell will survive, proliferate or exit the cell cycle and differentiate in response to soluble factors.

Addresses

Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center and the Sloan-Kettering Division, Graduate School of Medical Sciences, Cornell University, Box 216, 1275 York Avenue, New York, NY 10021, USA; e-mail: f-giancotti@ski.mskcc.org

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Abbreviations

ECM	extracellular matrix
ERK	extracellular signal regulated kinase
FAK	focal adhesion associated kinase
ILK	integrin-linked kinase
JNK	Jun amino-terminal kinase
LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase
PI-3K	phosphoinositide 3-kinase
PIP ₂	phosphatidylinositol 4,5-bisphosphate
SH	Src homology
TAM	tyrosine activation motif

Introduction

It has been known for a long time that normal cells need to adhere to serum-derived extracellular matrix (ECM) components, such as fibronectin and vitronectin, in order to proliferate *in vitro*. The loss of this requirement is a hallmark of neoplastic cells and represents perhaps the best correlate to *in vivo* tumorigenesis. These findings have led to the hypothesis that the ECM generates signals necessary for the progression of normal cells through the cell cycle and the speculation that these signals may be constitutively active in neoplastic cells (reviewed in [1]). Subsequent studies have revealed that, upon interacting with specific ECM components, some cell types exit from the cell cycle and undergo morphological and functional differentiation even in presence of otherwise mitogenic concentrations of growth factors. For example, while myoblasts proliferate and remain undifferentiated

on fibronectin, on laminin they cease growing and fuse to form myotubes. Endothelial cells display similar behavior: they proliferate on fibronectin, but are induced to form capillary-like structures when confronted with a laminin-rich basement membrane gel. Finally, fibronectin, which promotes proliferation of the above-mentioned cell types, promotes differentiation in erythroblasts (reviewed in [2]). These observations suggest that the ECM can have divergent effects on cellular behavior depending on its composition and the cell type involved.

Since their original discovery about a decade ago, it has become clear that the integrins not only mediate cell adhesion to the ECM, but also activate intracellular signaling pathways. Each integrin consists of an α and a β subunit, and in mammals we know of 16 distinct α subunits and 8 β subunits which variously combine to form 22 receptors, each characterized by a distinct, although largely overlapping, ligand-binding specificity (reviewed in [3,4]). Despite this high degree of apparent redundancy, most integrins seem to have specific biological functions during development (reviewed in [5]), raising the possibility of signaling differences between integrins.

In this review, I will focus on the membrane-proximal events and major signaling pathways activated by the ECM in mammalian fibroblasts and epithelial cells, and discuss the mechanisms by which these signaling events regulate cytoskeletal organization, progression through the G₁ phase of the cell cycle, cell survival, and cell differentiation.

Focal adhesion kinase

All $\beta 1$ and $\alpha \gamma$ subunit containing integrins share the ability to promote the assembly of focal adhesions and, at the same time, activate the focal adhesion associated kinase, FAK. FAK is an unusual nonreceptor tyrosine kinase which consists of a central catalytic domain flanked by amino-terminal and carboxy-terminal domains devoid of the Src homology (SH)2 or SH3 domains that are characteristic of other cytoplasmic tyrosine kinases. In contrast to its closest relative, Pyk2, which displays a restricted tissue distribution, FAK is widely expressed and also appears to be activated by those growth factor and cytokine receptors that affect the cytoskeleton (reviewed in [6]).

Activation

Although the mechanism by which integrins activate FAK is incompletely understood, it is clear that this process is tightly coupled to the process of assembly of focal adhesions and associated stress fibers. The

activation of FAK requires the same segment of the β integrin subunit cytoplasmic domain that is thought to interact with talin and mediate the incorporation of integrins in focal adhesions [7]. Talin binds directly to the carboxy-terminal domain of FAK [8], and also interacts with vinculin and thereby paxillin [9]. Paxillin in turn binds to a distinct site in the carboxyl terminus of FAK [10]. Thus, it is possible that the initial recruitment of FAK to activated integrins is indirect and mediated by talin. In apparent contrast with this hypothesis, it has been reported that the amino-terminal segment of FAK interacts directly *in vitro* with a portion of the integrin β subunit cytoplasmic domain that is distinct from the talin-binding site [11]. Figure 1 illustrates a model that reconciles these seemingly contradictory observations. Upon being recruited to nascent focal adhesions by talin, FAK would undergo a conformational change and interact through its amino-terminal domain with the integrin β subunit tail. As the amino terminus of FAK plays a negative autoregulatory role, possibly by folding back onto the catalytic domain [12], this conformational transition may be a prerequisite for FAK's catalytic activity. The aggregation of integrins that is a consequence of their binding to extracellular ligand would then result in a correspondent oligomerization of FAK. The kinase would finally be activated by a *trans*-autophosphorylation mechanism similar to that established for receptor tyrosine kinases. Although it needs validation, this model is appealing because it explains why the activation of FAK by stimuli such as lysophosphatidic acid (LPA), which acts on Rho and thereby enhances actin filament contractility, requires integrin ligation [13].

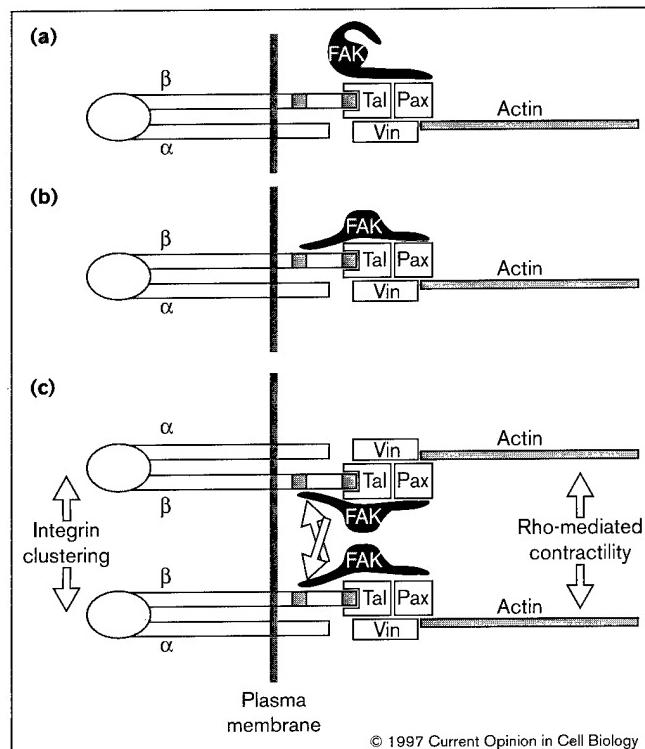
Target effectors

The activation of FAK has been linked to a number of molecular events. Activated FAK undergoes autophosphorylation at tyrosine residue 397 and thereby binds to the SH2 domain of Src or Fyn [14]. The Src family kinase (Src or Fyn) then phosphorylates a number of FAK-associated proteins, including paxillin, tensin and p130^{CAS} [15•,16]. While the phosphorylation of paxillin and tensin may be involved in the regulation of focal adhesions, p130^{CAS} is a multidomain docking protein and, upon phosphorylation, interacts with the adaptor proteins Crk and Nck, thereby potentially regulating signaling to the mitogen-activated protein kinases (MAPKs) extracellular signal regulated kinase (ERK) and Jun amino-terminal kinase (JNK) [16,17]. Src can also phosphorylate FAK at tyrosine residue 925, creating a binding site for the Grb2-mSOS complex, but the stoichiometry of this event appears to be low [18]. Finally, autophosphorylated FAK can combine with and activate phosphoinositide 3-kinase (PI-3K) [19]. FAK is thus linked to a number of intracellular signaling pathways that are potentially able to modify the cytoskeleton and influence transcriptional activity in the nucleus.

Role in cell migration and cell survival

What is the biological function of FAK? Although there is evidence suggesting that FAK can, upon overexpression

Figure 1



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Model of FAK recruitment and activation. (a) The binding of ligand (shaded oval) to the integrin (α and β) ectodomain promotes, presumably via a conformational change, the interaction of the β integrin subunit tail with talin (Tal) and, via talin, with vinculin (Vin) and paxillin (Pax). Vinculin and paxillin interact with the actin cytoskeleton. FAK is recruited to the nascent complex via the interaction of its carboxy-terminal domain with paxillin and talin. (b) FAK undergoes a conformational change which allows it to interact through its amino-terminal domain with a membrane-proximal segment of the β integrin tail. (c) The geometry of integrin-binding sites in the ECM and Rho-mediated contractility cooperate to promote integrin clustering, thereby bringing different FAK molecules together. FAK then undergoes *trans*-autophosphorylation (indicated by crossed arrows). The shaded squares in the integrin β subunit tail correspond to the FAK- and talin-binding sites.

or association with elevated levels of Src, activate the Ras-ERK signaling pathway in response to cell adhesion [12,17], it is unclear if FAK physiologically plays this role. In fact, recent studies have provided evidence that, in normal fibroblasts and endothelial cells, the activation of ERK in response to integrin ligation is mediated by the adaptor protein Shc independently of FAK [20•]. Accordingly, the introduction of a dominant-negative version of FAK does not impair the activation of ERK caused by cell adhesion to fibronectin in these cells [21•]. Although these findings do not support the hypothesis that FAK can signal through ERK in normal cells, it remains possible that FAK plays such a role upon overexpression in carcinomas [22].

The analysis of FAK knockout mice has provided important information on the biological function of FAK.

Embryonic fibroblasts derived from these mice form numerous centrally located small focal contacts, but fail to form the large peripheral focal adhesions that may be required at the leading edge of the cell to propel migration; accordingly, these cells migrate less efficiently than control cells [23]. The observations that overexpression of FAK increases cell migration [24] while a dominant-negative form of the kinase inhibits it [25] are also consistent with a role of FAK in the dynamic regulation of focal adhesions during cell migration. Finally, recent data have indicated that a constitutively active form of FAK can promote anchorage-independent survival and growth in epithelial cells [26*], suggesting that FAK may also contribute to cell survival and proliferation.

Other membrane-proximal kinases

Integrin signaling is likely to involve other kinases in addition to FAK and Src family kinases. A recent report has provided evidence that cell adhesion to various ECM components activates Abl, a tyrosine kinase with different functions in the cytoplasm and in the nucleus [27]. Cytoplasmic Abl is likely to be incorporated in focal adhesions through its interaction with Mena, a homolog of the *Drosophila* Abl substrate Ena [28**]. As Mena contains a central proline-rich segment, which binds to profilin and may be involved in the assembly of actin filaments, Abl-mediated phosphorylation of Mena may contribute to the regulation of focal adhesions. It has also been shown that cell adhesion to the ECM is necessary for the transport of Abl from the cytoplasm to the nucleus at the G₁→S-phase boundary of the cell cycle [27]. As nuclear Abl is potentially involved in regulating the transcription of genes important for S-phase entry, this may provide a mechanism for cell cycle control by the ECM.

In contrast to FAK and Abl which are activated in response to cell adhesion, the activity of the serine/threonine-specific integrin-linked kinase (ILK) appears to be inhibited in response to integrin ligation [29*]. ILK was originally isolated by virtue of its ability to interact with the integrin β1 cytoplasmic domain in the yeast two-hybrid system and was later shown to be localized to focal adhesions [29*]. Interestingly, the amino-terminal segment of ILK contains a series of ankyrin-like repeats which may mediate association with the actin cytoskeleton, while the carboxy-terminal catalytic domain includes the region that interacts with the β1 cytoplasmic tail *in vitro*. Overexpression of ILK leads to constitutive activation of ERK and anchorage-independent cell growth [30]. However, adhesion to fibronectin decreases the activity of ILK in cells that express physiological levels of the kinase [29*]. It is thus unlikely that ILK mediates the activation of ERK and stimulation of cell cycle progression observed in response to the ECM. Rather, ILK appears to be a novel type of transforming kinase negatively regulated by cell adhesion.

Transmembrane adaptors

Recent studies have indicated that integrins can associate with different classes of transmembrane adaptors. The αvβ3 and αIIbβ3 integrins are physically and functionally associated with the integrin-associated protein (IAP), which consists of an extracellular immunoglobulin domain followed by five transmembrane helices and a short cytoplasmic tail [31]. A recent study has indicated that IAP not only cooperates with β3 integrins in binding to thrombospondin, but also activates a heterotrimeric Gi protein dependent intracellular pathway that leads to the activation of the tyrosine kinase Syk and its association with FAK [32]. These findings suggest that IAP may function in concert with β3 integrins to regulate intracellular signaling in response to thrombospondin.

The α3β1 and α6β1 integrins combine with the tetraspan proteins CD9, CD63 and CD81, which are characterized by four transmembrane segments [33]. Interestingly, CD63 and CD81 have been shown to be associated with a type II phosphatidylinositol 4-kinase (PI-4K) and may therefore control the first step in the biosynthesis of phosphatidylinositol 4,5-bisphosphate (PIP₂) in response to cell adhesion [34].

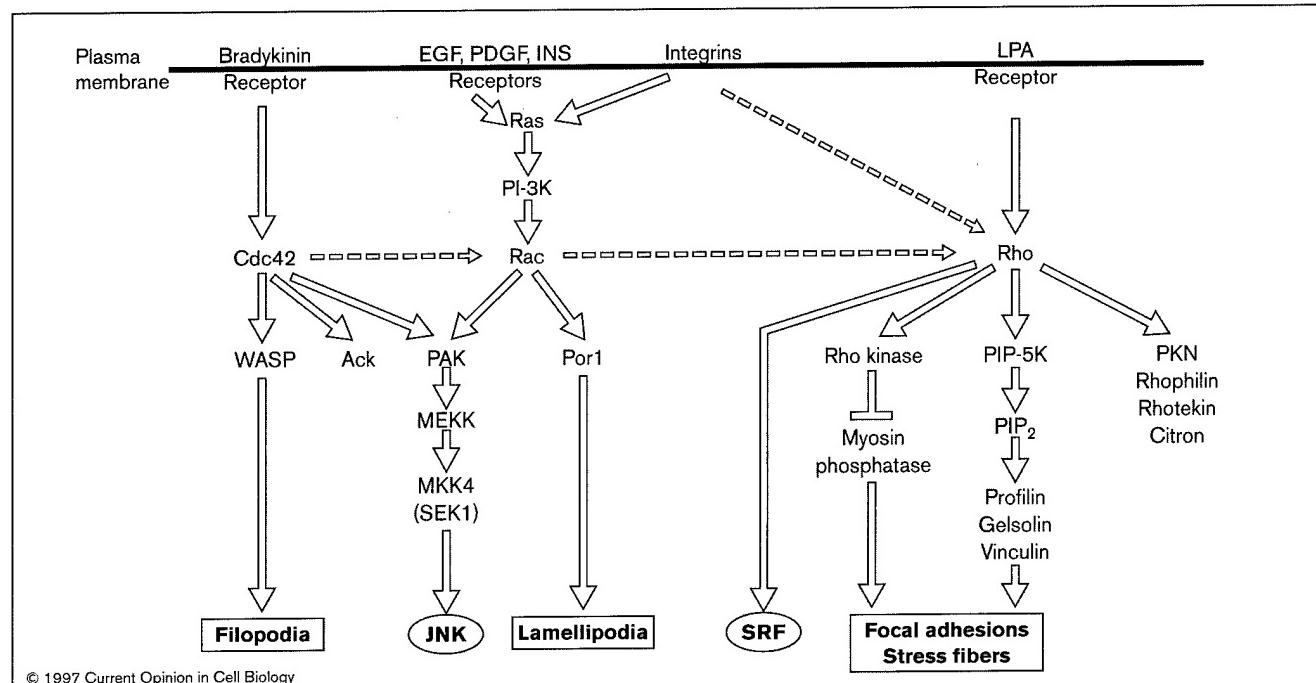
Finally, it has been observed that the two-transmembrane-domain adaptor caveolin is constitutively associated with β1 integrins in resting cells and becomes associated with the adaptor protein Shc in response to integrin ligation [20**]. Taken together, these observations suggest that various transmembrane adaptors link specific integrins to distinct intracellular signaling pathways.

Rho family GTPases

Rho family GTPases regulate the actin cytoskeleton and influence gene expression by interacting with multiple distinct target effectors (reviewed in [35]) (Figure 2). Cdc42 has been implicated in the formation of filopodia in response to bradykinin; Rac promotes the establishment of lamellipodia in response to epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin; and Rho is involved in the organization of focal adhesions and associated stress fibers in response to LPA or bombesin. In addition to these morphological effects, Rho family proteins activate MAPK cascades involved in the control of immediate early gene expression: Cdc42 and Rac are known to activate JNK, and Rho activates the serum-response factor (SRF). Finally, there is evidence that the Rho family proteins are horizontally interconnected in a hierarchical fashion: Cdc42 activates Rac, and Rac activates Rho.

In addition to being necessary for the morphological changes caused by Rho family proteins in response to growth factors [13], integrin ligation is sufficient to activate at least some of the pathways controlled by Rac and Rho. Two lines of evidence support the notion that integrins can activate Rho. First, it has been known for some time

Figure 2



Signaling via Rho family GTPases. The major signaling pathways involving Rho proteins (Cdc42, Rac and Rho), recently reviewed elsewhere [35], are illustrated diagrammatically. The integrins can activate Rac via Ras and PI-3K, and Rho through mechanisms that remain to be determined. Cdc42, Rac and Rho, which are 'horizontally' interconnected in a hierarchical fashion, interact with a number of target effectors. Some of these, such as WASP, Por1, Rho kinase and PIP-5K, are responsible for organizing cytoskeletal structures such as filopodia, lamellipodia and focal adhesions. Others activate elements, such as JNK and SRF, that influence immediate early gene expression. The functions of Ack, PKN, Rhophilin, Rhotekin and Citron remain to be fully established. Ack, activated Cdc42-binding kinase; EGF, epidermal growth factor; INS, insulin; MEK (not shown), MAPK/ERK kinase; MEKK, MEK kinase/SAPK kinase kinase; MKK/SEK, SAPK kinase; PAK, p21(Cdc42/Rac)-activated kinase; PDGF, platelet-derived growth factor; PIP-5K, phosphatidylinositol phosphate 5-kinase; PKN, protein kinase N; Por1, partner of Rac 1; SAPK, stress-activated protein kinase (also known as JNK); SRF, serum-response factor; WASP, Wiskott-Aldrich syndrome protein. Dotted arrows indicate pathways for which the biochemical evidence is less strong. Ovals and rectangles distinguish between molecules and structures, respectively.

that integrins can stimulate the production of PIP₂ and recent studies have provided evidence that this effect is mediated by Rho [36], possibly through its interaction with a type I isoform of phosphatidylinositol 4-phosphate 5-kinase (PIP4-5K) [37]. Second, dominant-negative Rho can partially suppress the activation of ERK in response to ligation of the $\alpha 5\beta 1$ and $\alpha 6\beta 4$ integrins [38,39], suggesting that a Rho-regulated pathway may be necessary for full activation of ERK upon cell adhesion.

The observation that Rho activates a PIP4-5K [36] is intriguing. In addition to being a substrate for both PI-3K and phospholipase C- γ , and therefore being necessary for the generation of crucial lipid second messengers in response to growth factor stimulation, PIP₂ (produced from phosphatidylinositol 4-phosphate by PIP4-5K) binds to vinculin and promotes its interaction with both talin and actin [40••]. Furthermore, PIP₂ can induce the release of actin monomers from profilin and gelsolin complexes (reviewed in [41]). These events are likely to participate in the assembly of actin filaments at focal adhesions and other sites of interaction with the ECM. Rho can

also activate a serine/threonine protein kinase that is homologous to myotonic dystrophy kinase, namely Rho kinase, which plays an important role in the assembly of focal adhesions [42•]. This kinase phosphorylates the myosin-binding subunit (MBS) of myosin phosphatase, thereby suppressing the activity of the enzyme [43•]. The resulting net increase in myosin phosphorylation is expected to promote actomyosin contractility and participate in the lateral association of nascent focal complexes, an event which has been suggested to be critical for the activation of FAK [44]. These results are consistent with the previous observation that Rho is required upstream of FAK and clarify some of the mechanisms by which Rho would promote the assembly of focal adhesions and stress fibers (Figure 1).

Recent studies have provided evidence for an involvement of Rac in integrin signaling. In particular, it has been shown that ligation of the $\alpha 5\beta 1$ and $\alpha 6\beta 4$ integrins causes activation of JNK [39•,45]. In the case of $\alpha 6\beta 4$ integrin, this event is suppressed by dominant-negative forms of Ras and Rac and by the PI3-K inhibitor wortmannin. As

PI3-K is a target effector of Ras and an activator of Rac [46•], it would appear that the activation of JNK by $\alpha 6\beta 4$ integrin is mediated by the sequential activation of Ras, PI3-K and Rac. It is likely that the same and possibly additional mechanisms link $\alpha 5\beta 1$ and other integrins to the activation of Rac.

The ability of integrins to control Rho family dependent pathways and thereby organize the cytoskeleton may be critical for the control of cell proliferation. Although Rho family proteins regulate immediate early gene expression, recent studies have shown that it is their ability to organize the cytoskeleton that best correlates with cell cycle progression [47••,48••]. Perhaps, as suggested by a very recent study [49], a certain degree of cytoskeletal organization is required to orient the signal transduction machinery of the cell in such a way that it can respond to biochemical signals from the ECM and soluble mitogens.

Role of $\alpha 6\beta 4$ integrin signaling in assembly of hemidesmosomes

In contrast to $\beta 1$ and αv integrins which participate in the formation of focal adhesions, the $\alpha 6\beta 4$ integrin, which is characterized by the uniquely large cytoplasmic domain of the $\beta 4$ subunit, is involved in the assembly of hemidesmosomes. The hemidesmosomes are punctuate junctions that stabilize the adhesion of stratified and complex epithelia to the basement membrane. In contrast to focal adhesions, which are linked to the actin cytoskeleton, the hemidesmosomes are connected to the keratin filament system. The nucleation of hemidesmosomes is triggered by the binding of $\alpha 6\beta 4$ integrin to laminin 5 and mediated by a signaling mechanism. Ligation of $\alpha 6\beta 4$ integrin activates an integrin-associated kinase and thereby induces tyrosine phosphorylation of the $\beta 4$ cytoplasmic tail [50]. This event is followed by the sequential recruitment of the adaptor molecules Shc and Grb2, which link $\alpha 6\beta 4$ integrin to Ras signaling. Later on, $\alpha 6\beta 4$ integrin becomes incorporated in hemidesmosomes. The β tail contains towards its carboxyl terminus two pairs of type III fibronectin-like modules interrupted by a 142 amino acid long connecting segment (Figure 3). The multiple $\beta 4$ tyrosine residues that are phosphorylated *in vivo* include a tyrosine activation motif (TAM) located in the connecting segment. Interestingly, phenylalanine substitutions at the $\beta 4$ TAM disrupt the association of $\alpha 6\beta 4$ integrin with hemidesmosomes, but do not affect recruitment of Shc and Grb2, suggesting that the $\beta 4$ TAM plays a role in the assembly of hemidesmosomes, but not the recruitment of Shc and Grb2 [50] (Figure 3).

The TAM is a bidentate phosphorylation motif consisting of two closely spaced tyrosine residues followed by a leucine at position +3, and was originally identified in the cytoplasmic tails of signaling subunits of immune receptors. It is thought that, upon phosphorylation, the immune receptor TAMs bind to the two tandem SH2 domains of tyrosine kinases, such as Syk and ZAP70, which mediate

subsequent downstream signaling events (reviewed in [51]). It is possible that the phosphorylation of the $\beta 4$ integrin TAM, which is a transient event, regulates the assembly of hemidesmosomes by an analogous signaling mechanism [52]. In this model, the $\beta 4$ TAM mediates the recruitment of an SH2-SH2-domain adaptor, kinase, or phosphatase which directly or indirectly modifies cytoskeletal element(s) of hemidesmosomes, enabling them to bind to a different region in the $\beta 4$ integrin tail. This hypothesis would also explain the previous observation that the incorporation of $\alpha 6\beta 4$ integrin into hemidesmosomes requires not only the $\beta 4$ TAM but also the two type III fibronectin-like modules upstream of the connecting segment [53].

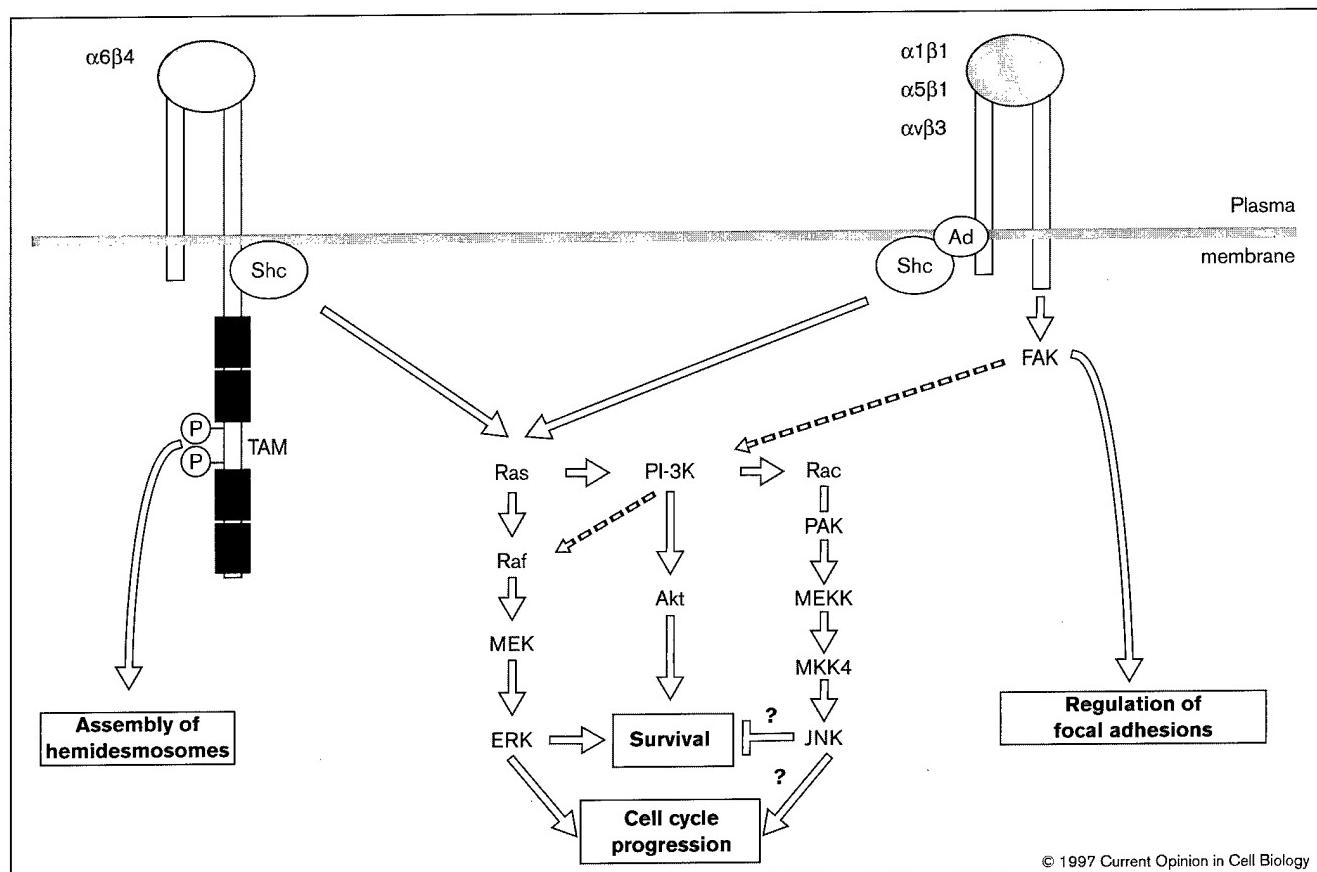
The adaptor Shc links a group of integrins to the Ras-ERK signaling cascade

Despite some initial controversy [54,55], it is now generally accepted that the activation of ERK in response to integrin ligation requires Ras signaling [12,20••]. What is the mechanism by which integrins activate Ras? Recent studies have provided evidence that certain integrins, which include the laminin receptor $\alpha 6\beta 4$, the laminin/collagen receptor $\alpha 1\beta 1$, the fibronectin receptor $\alpha 5\beta 1$ and the broad specificity RGD (Arg-Gly-Asp)-binding receptor $\alpha v\beta 3$, are linked to the Ras-ERK signaling pathway and the control of immediate early gene expression by the adaptor protein Shc. Other integrins, in contrast, appear to be unable to activate Ras signaling [20••,39•,50]. Shc is an SH2 and phosphotyrosine binding (PTB) domain adaptor protein which links various tyrosine-phosphorylated signal transducers to Ras. Upon recruitment to activated receptors, Shc is phosphorylated on tyrosine and binds to the Grb2-mSOS complex. This process results in the juxtaposition of the GTP exchange factor domain of mSOS to its target Ras, leading to its activation (reviewed in [56]). Although there is evidence that Shc can bind directly to the tyrosine-phosphorylated cytoplasmic domain of $\beta 4$ integrin, the recruitment of Shc to activated $\beta 1$ and αv integrins appears to be indirect and mediated by the interaction of the integrin α subunit with a transmembrane adaptor, possibly caveolin (Figure 3).

Control of cell cycle progression

The association of specific integrins with Shc is important for activation of the Ras-ERK signaling pathway in response to cell adhesion, while FAK plays a minor role in this process. In fact, ligation of $\alpha 1\beta 1$, $\alpha 5\beta 1$, and $\alpha v\beta 3$ integrins, which are linked to Shc, results in ERK activation, but ligation of other integrins does not produce this effect, even though FAK is stimulated [20••]. In addition, cross-linking of a mutant single-chain $\alpha 1$ integrin subunit causes recruitment and tyrosine phosphorylation of Shc and activation of ERK without inducing FAK activation [20••]. Finally, a dominant-negative version of Shc suppresses ERK activation in response to integrin ligation [20••], but two distinct dominant-negative versions of FAK do not ([21•]; KK Wary, FG Giancotti, unpublished

Figure 3



Integrin-mediated signaling pathways. Cell survival and cell cycle progression are controlled by pathways that are largely distinct from those that are involved in the assembly of adhesive junctions such as focal adhesions or hemidesmosomes. The $\alpha 6\beta 4$ integrin (left) regulates the assembly of hemidesmosomes by a mechanism which includes phosphorylation of a TAM sequence in the $\beta 4$ tail and promotes progression through the G₁ phase of the cell cycle via recruitment of Shc and activation of Ras-ERK signaling. Although all $\beta 1$ and αv integrins (right) can regulate focal adhesions via FAK, only some of them, including those indicated in the figure, can recruit Shc, activate Ras-ERK signaling, and promote cell cycle progression. In this case the recruitment of Shc is mediated by a transmembrane adaptor, probably caveolin. Cell survival is promoted by integrins via ERK and/or Akt. JNK may inhibit cell survival if ERK is not activated or may cooperate with ERK to promote cell cycle progression, but this hypothesis needs validation. Ad, transmembrane adaptor, possibly caveolin; MEK, MAPK/ERK kinase; MEKK, MEK kinase/SAPK kinase; MKK4, SAPK kinase; PAK, p21(Cdc42/Rac)-activated kinase; SAPK, stress-activated protein kinase (also known as JNK). Circled P, phosphorylation; gray oval, integrin ligand. Dotted arrows indicate pathways for which the biochemical evidence is less strong.

data). These results indicate that Shc is necessary and sufficient to link specific integrins to the Ras-ERK signaling pathway. However, they do not exclude the possibility that FAK may cooperate with Shc to fully activate ERK in response to cell adhesion.

Two recent studies suggest a potential mechanism by which FAK could effect signaling to ERK. In the first study, it was shown that full activation of Raf, but not Ras, by mitogenic growth factors requires integrin ligation [57••]. This suggests that the activation of an integrin-dependent pathway impinging on Raf is necessary for ERK activation in response to growth factor stimulation. The second study showed that integrin ligation causes a significant activation of PI3-K, which is necessary for the activation of Raf by Ras in response to cell adhesion [58••]. Although the mechanism of PI3-K activation by integrins has not yet been fully explored, FAK may play a role

in this process and thereby participate in rendering Raf responsive to Ras.

What is the biological role of the activation of Ras-ERK signaling by specific integrins? The studies on Shc signaling mentioned above have indicated that, in primary cells, engagement of integrins linked to Shc activates transcription from the Fos serum-response element (SRE) and promotes progression through the G₁ phase of the cell cycle in response to growth factors. In contrast, ligation of other integrins does not stimulate SRE-dependent transcription and results in cell cycle arrest even in the presence of otherwise mitogenic concentrations of growth factors [20••]. These observations suggest that a simultaneous stimulation of Ras by integrins and growth factor receptors is needed to reach a threshold level of MAPK activation required for optimal transcription of Fos. As integrin ligation also activates JNK, it is likely that cell

adhesion will also activate Jun. Fos-Jun heterodimers can then regulate transcription of genes necessary for cell cycle progression [59] (Figure 3).

Recent studies have indicated that cell adhesion is specifically required for the induction of cyclin D1 and for the activation of the cyclin E-cdk2 (cyclin-dependent kinase 2) complex in early-mid G₁ phase [60^{**},61^{**}]. Although cyclin D1 is regulated by cell adhesion at both the transcriptional and the translational levels, the effect of cell adhesion on cyclin E-cdk2 activity appears to be indirect and mediated by a downregulation of the cdk inhibitors p21 and p27. These findings suggest several mechanisms by which integrin signaling could regulate the cell cycle. In fact, the cyclin D1 promoter contains an SRE and a c-ets site, both of which are targets of ERK signaling [62]. In addition, it has been recently shown that cell adhesion to the ECM causes activation of p70S6K [63,64], a kinase that may regulate cyclin D1 translation and p27 levels by multiple mechanisms (reviewed in [65]).

Regulation of cell migration

A recent study has provided evidence that the activation of ERK in response to integrin ligation may also play a role in regulating cell migration [66^{*}]. In fact, ERK activation by a constitutively active form of the MAPK kinase MEK1 increases cell migration, while ERK inhibition by antisense oligonucleotides or a specific chemical inhibitor reduces cell migration [66^{*}]. Interestingly, the ability of ERK to stimulate cell migration correlates with the phosphorylation and activation of myosin light chain kinase (MLCK). In addition, *in vitro* assays indicate that ERK can phosphorylate MLCK, and ERK-phosphorylated MLCK displays an enhanced capacity to phosphorylate its physiological substrate, the myosin light chain, in a calmodulin-dependent manner. These findings suggest that ERK may regulate cell migration by stimulating actomyosin contractility.

Multiple integrin-dependent pathways are likely to be involved in protection from apoptotic cell death

Normal cells denied anchorage to an appropriate extracellular matrix undergo apoptosis and this may be a mechanism to insure that cells which are displaced from their natural environment are eliminated. Tumor cells appear to be unusually resistant to this homeostatic mechanism, which may explain their propensity to home within tissues different from their own (see Frisch and Ruoslahti, this issue, pp 701–706).

The ability of the ECM to promote cell survival appears to be mediated by at least two distinct signaling pathways. The studies on the role of Shc in integrin signaling have indicated that, in primary cells, adhesion mediated by integrins not linked to Shc results not only in cell cycle arrest, but also in apoptotic cell death [20^{**}]. In accordance with a critical role of Shc signaling in

protection from apoptosis, a dominant-negative form of Shc induces apoptotic death in primary endothelial cells plated on fibronectin in presence of mitogens (KK Wary, FG Giancotti, unpublished data). Although not demonstrated, the integrins that combine with Shc may promote cell survival by elevating the activity of ERK.

Other studies have shown that constitutively active forms of FAK and PI3-K can protect cells from suspension-induced apoptosis [26^{*},67^{*}]. Although the anti-apoptotic effect of PI3-K has been shown to be mediated by the serine/threonine kinase Akt, the mechanism by which integrins activate PI3-K remains to be established, but probably involves FAK. Alternatively, as PI3-K is a major target effector of Ras [46^{*}], the activation of PI3-K by integrins could be mediated by the ability of Shc to activate Ras. If the first hypothesis is correct, then one could envision a model in which all integrins activate the FAK-PI-3K-Akt pathway, but only a subset is capable of activating the Shc-Ras-ERK pathway. This would imply that both pathways need to be activated for survival (Figure 3).

ECM-induced exit from the cell cycle may facilitate differentiation

It has been known for long that adhesion to an appropriate ECM is required for differentiation in a number of cell types. The studies on Shc-mediated signaling indicate that ECM recognition by integrins that fail to activate Shc results in cell cycle exit even in the presence of mitogens [20^{**}]. As withdrawal from the cell cycle is a prerequisite for differentiation, it is possible that those integrins which do not activate the Shc pathway may promote differentiation. This hypothesis is consistent with a number of previous observations. For example, in endothelial cells, adhesion to fibronectin, which is mediated by the Shc-linked integrin $\alpha 5\beta 1$, promotes proliferation [20^{**}], but adhesion to a laminin-rich matrix, which is mediated by the non-Shc-linked $\alpha 2\beta 1$ integrin, promotes exit from the cell cycle and formation of capillary-like structures [68]. Similarly, myoblasts proliferate on fibronectin but fuse to form myotubes on laminin, and these two opposing functions have been linked to the expression of $\alpha 5\beta 1$ and $\alpha 6\beta 1$ integrin, respectively [69^{*}].

The ability of the ECM to promote exit from the cell cycle may be necessary, but is unlikely to be sufficient to induce differentiation. In fact, although primary mammary epithelial cells need to adhere to a basement membrane gel in order to activate transcription of tissue-specific genes, such as β -casein and lactoglobulin, this effect requires simultaneous exposure to lactogenic hormones [70]. A recent study has provided evidence that the lactogenic hormone prolactin promotes the binding of the transcription factor Stat5 to γ -interferon activation site (GAS)-related DNA sequence elements in the β -lactoglobulin promoter in breast epithelial cells plated on laminin, but not on collagen or poly-L-lysine [71].

Taken together, these findings suggest that, although interaction with a specific ECM may be sufficient to promote exit from the cell cycle, complete morphological and functional differentiation is likely to require the integration of signaling pathways activated by both integrins and soluble differentiation factors.

Conclusions and future directions

The studies reviewed above indicate that the integrins that are linked to Shc-mediated signaling cooperate with growth factor receptors to stimulate cell proliferation, while the remaining integrins may passively, or perhaps actively, enable exit from the cell cycle and thereby facilitate cell differentiation. There is already evidence pointing to other signaling differences between integrins [32,33,72,73], and future studies will probably reveal additional integrin-specific pathways.

An important conclusion of many studies is that the signaling pathways activated by growth factor receptors and integrins are extensively interconnected, most importantly at the level of Ras, PI-3K and FAK. In addition, there is evidence that certain integrins form physical complexes with growth factor receptors [74,75•]. Future studies will have to address the molecular mechanisms that regulate the integration and, ultimately, the interpretation of signals from integrins and growth factor receptors. In particular, many signaling intermediates interact with, and receive signals from, various upstream elements as well as bind to a large number of downstream target effectors. It will be interesting to dissect which molecular pathways are really converging and diverging at each one of these nodes in response to signals from the ECM and growth factors.

We have just begun to understand how all these pathways influence cell survival, proliferation, and differentiation. Although some membrane-proximal events have been delineated, it will be important to identify the nuclear events controlled by these pathways and understand how these events in turn control the cell cycle and apoptotic machinery. Finally, an analysis of the molecular mechanisms that allow tumor cells to survive and proliferate in the absence of proper signals from the ECM will be an important area of investigation.

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